STUDIES IN CHOLESTEROL METABOLISM IN THE ANIMAL BODY

by

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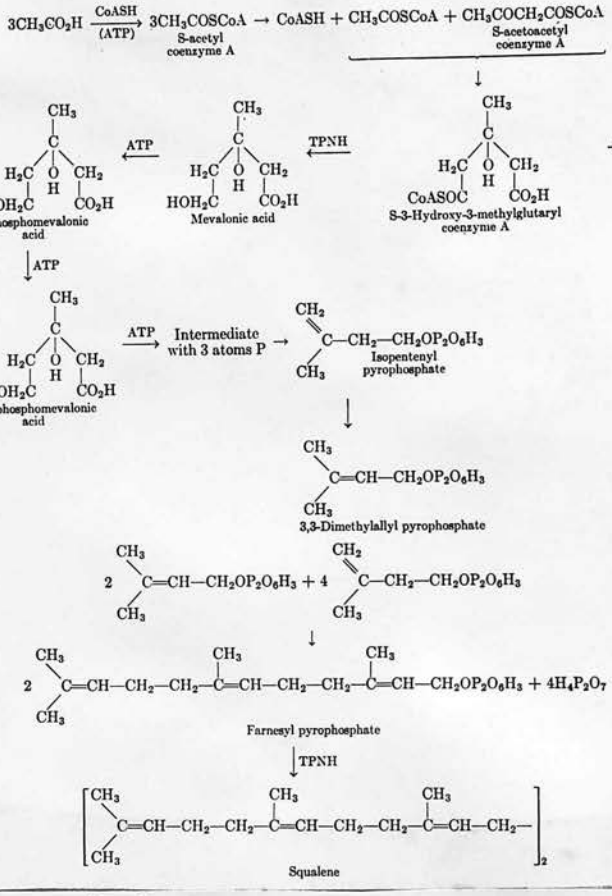
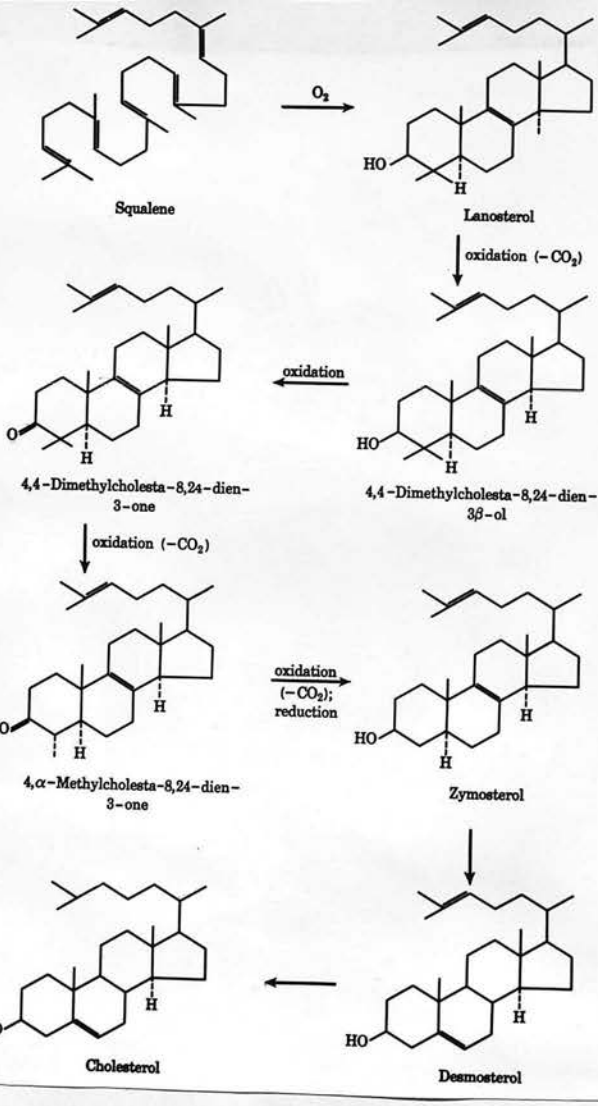
CONTENTS

INTRODUCTION

FIG. 1


3CH3CO2H
CoASH (ATP)
3CH3COSCoA
S-acetyl coenzyme A
CoASH + CH3COSCoA + CH3COCH2COSCoA
S-acetoacetyl coenzyme A
+ CoASH
S-3-Hydroxy-3-methylglutaryl coenzyme A
TPNH
Mevalonic acid
ATP
5-Phosphomevalonic acid
ATP
5-Diphosphomevalonic acid
ATP
Intermediate with 3 atoms P
Isopentenyl pyrophosphate
3,3-Dimethylallyl pyrophosphate
Farnesyl pyrophosphate
TPNH
Squalene


Squalene
O2
Lanosterol
oxidation (−CO2)
4,4-Dimethylcholesta-8,24-dien-3-one
oxidation
4,4-Dimethylcholesta-8,24-dien-3β-ol
oxidation (−CO2)
4,α-Methylcholesta-8,24-dien-3-one
oxidation (−CO2); reduction
Zymosterol
Cholesterol
Desmosterol

Cholesterol, the most ubiquitous sterol in the animal body, is probably present in every cell, yet despite its long biochemical history (it was isolated by Chevreul in 1816), very little is known of its function.

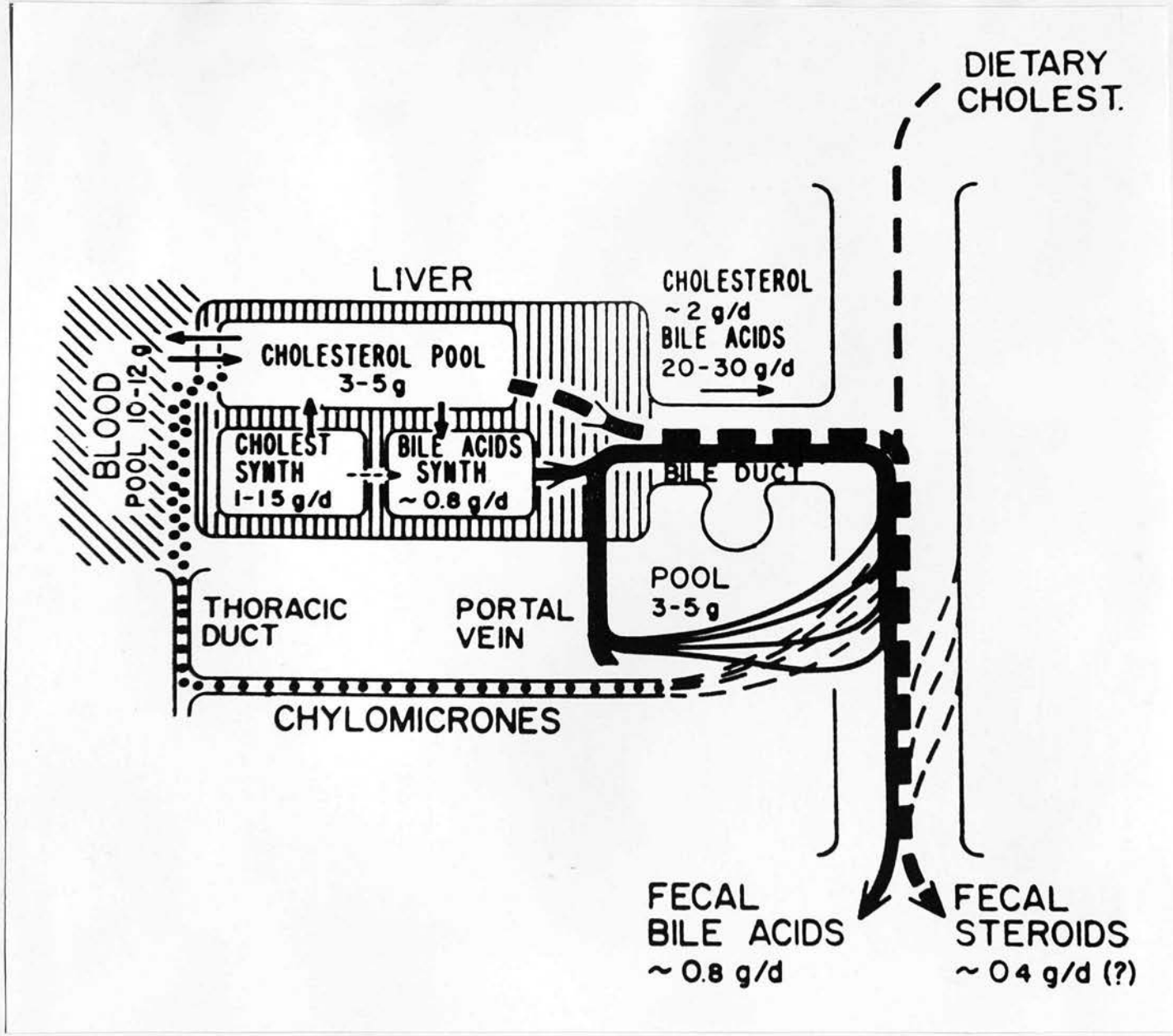
The structure of cholesterol is based on the steroid nucleus, cyclopentano-perhydro-phenanthrene. This, on addition of a hydrocarbon side chain containing eight carbon atoms, becomes cholestane. Cholesterol is cholest-5-en-3β-ol (see Figure 1).

Cholesterol is found in the membrane of all cells where it may contribute to the selectively permeable nature of this structure; it is also found in nervous tissue where it is extremely inert and is thought to function as an insulator (Minovici, 1927).

About 10% of the cholesterol in the body is found in the blood, largely in the form of plasma lipoproteins, though a little is present in the stroma of the red cells. This circulating cholesterol is metabolically active and its concentration is under homeostatic control.

Cholesterol present in the body may have been absorbed intact from food in the intestine, or may have been biosynthesized by the animal

FIG. 2


DIETARY CHOLEST.
LIVER
CHOLESTEROL ~2 g/d
BILE ACIDS 20-30 g/d
CHOLESTEROL POOL 3-5 g
BLOOD POOL 10-12 g
CHOLEST SYNTH 1-15 g/d
BILE ACIDS SYNTH ~0.8 g/d
BILE DUCT
POOL 3-5 g
THORACIC DUCT
PORTAL VEIN
CHYLOMICRONES
FECAL BILE ACIDS ~0.8 g/d
FECAL STEROIDS ~0.4 g/d (?)

itself (see Figure 2). Mammalian liver contains all the enzymes necessary for the synthesis, and most investigations have been carried out using this organ, although virtually all nucleated cells can also perform the synthesis.

Cholesterol Biosynthesis

The process of cholesterol biosynthesis has only been described in detail within the last decade, and deservedly has been the subject of much attention, whereas studies of cholesterol degradation appear to have been much less spectacular.

In 1937, Rittenberg and Schoenheimer showed that the label from deuterated water was incorporated into cholesterol in the mouse indicating that the latter could be formed from small molecule precursors. Five years later, Bloch and Rittenberg (1942) showed that the two carbon atoms of acetate could provide all the carbon of cholesterol. From studies of the distribution of isotope in cholesterol biosynthesized from labelled acetate, Bloch proposed that sterol synthesis might be related to that of the terpenes and might involve the isoprene

structure as a basic unit. This proposal revived interest in a hypothesis proposed much earlier by Channon (1926) to the effect that squalene, a triterpenoid molecule, might be a biochemical precursor of the steroid nucleus. Robinson, in 1934, suggested a method of rearranging the squalene formula so as to form a steroid on cyclization. In 1953, Langdon and Bloch isolated labelled squalene from the livers of mice after the injection of labelled acetate; they then fed this squalene to rats and showed it was converted to cholesterol. Together with Woodward (1953), Bloch suggested a theoretical method of folding the squalene molecule which differed from that of Robinson. Some very elegant degradative studies by Cornforth and Popjak (1954) made it possible to ascertain the origin of each atom of the cholesterol molecule with reference to the labelled acetate precursor, and their findings confirmed the Woodward-Bloch hypothesis.

Little was still known of the stages between acetate and squalene, but in 1956 Tavormina, Gibbs and Huff found that mevalonic acid (MVA, β-hydroxy-β-methyl-5-valerolactone) could function as an efficient precursor of cholesterol. This

compound can be formed by the condensation of three acetyl-CoA molecules.

After activation to the pyrophosphate, mevalonic acid can form squalene, probably via an intermediate compound such as farnesinic acid (see Figure 1). An early stage following the cyclization of squalene is thought to involve lanosterol, which possesses three more angular methyl groups than does cholesterol, has a double bond between C atoms 8 and 9, and has a more saturated side chain, so that even after the cyclization step several modifications in structure are needed.

The process of biosynthesis can be affected at different stages by the actions of various compounds. There appears to be a type of feed-back control mechanism so that the feeding of intermediates such as MVA or squalene or of cholesterol itself, results in a lowering of hepatic cholesterogenesis (Morris, Chaikoff, Felts, Abraham and Fansah, 1957; Langdon and Bloch, 1953). The site of the control mechanism has been narrowed to the stages involved in the reduction of hydroxy-methyl-glutaryl-CoA to MVA.

Since, however, many of the stages in the

pathway are not confined only to the biosynthesis of cholesterol, interference with the earlier steps may affect the synthesis of a variety of compounds, and thus the results may be difficult to interpret when only applied to cholesterol.

The type of fat in the diet appears to affect cholesterol biosynthesis. Several groups of investigators have found that the feeding of unsaturated fat to rats stimulated synthesis (Mukherjee and Alfin-Slater, 1958; Wood and Migicovsky, 1958; Avigan and Steinberg, 1958). Linazasoro, Hill, Chevalier and Chaikoff (1958) failed to observe such a result.

Regulation of biosynthesis is also affected by some humoral agents. Hyperthyroidism or the administration of thyroid hormones causes an increase in hepatic synthesis, whereas the effect of the oestrogens is to depress it. No satisfactory explanation has yet been produced.

Other factors affecting cholesterol biosynthesis include the detergent Triton which stimulates the process, as do certain metallic ions, namely chromous, chromic, manganous and ferric, but ferrous, vanadous and cobaltous ions have an inhibitory effect (Curran, 1954). Other inhibitory factors are compounds such as 2-phenyl-

butyric acid, which prevent the acetylation of coenzyme A. Decreased cholesterol production has been shown to follow the administration of MER 29[x], which apparently inhibits one of the latter stages of synthesis following the cyclization of squalene, with the resultant accumulation of one of the intermediate sterols, desmosterol (24-dehydro-cholesterol).

Degradation of Cholesterol

From a quantitative point of view the only important catabolic reaction undergone by cholesterol is the conversion in the liver to bile acids which accounts for about 80% of the cholesterol degraded daily (Siperstein and Murray, 1955).

Labelled cholesterol has been shown to be converted to all the steroid hormones. The major hormone forming tissues (adrenals, testes and ovaries) have been shown to contain an enzyme which can remove the terminal iso-caproyl group from cholesterol, leaving the C-21 steroid, pregn-5-en-3-ol-20-one, which can act as precursor of all the steroid hormones (Lynn, Staple and Gurin, 1954; Zaffaroni, Hechter and Pincus, 1951).

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[x](1-[4-diethyl-aminoethoxy] phenyl-1-[p-tolyl]-2-[p-chlorophenyl] ethanol)

The conversion of cholesterol to cholic acid, the parent bile acid, requires several modifications of its structure. These include: inversion of the C_3-hydroxyl group, reduction of the 5-6 double bond, introduction of additional hydroxy groups on the nucleus and the oxidative removal of the three terminal carbon atoms. The details of this conversion, knowledge of which is far from complete, will be discussed in the Introduction to Section 2.

The extent of degradation of cholesterol to bile acids seems to be controlled by a feedback control mechanism in a similar way to the synthetic process. If biliary excretion is stimulated by the creation of a bile fistula, the production of bile acids is increased (Eriksson, 1957). The feeding of bile acids raises serum cholesterol levels, presumably by decreasing the catabolism of cholesterol as a result of the feedback mechanism (Swell, Flick, Field and Treadwell, 1953).

Other factors which have been shown to affect bile acid production are the fat composition of the diet, nicotinic acid and certain metallic ions. <u>In vitro</u> oxidation of

cholesterol by rat liver mitochondrial preparations has been shown to increase when the animals had been pre-fed ferric, cobalt or nickel ions or nicotinic acid (but not nicotinamide) (Kritchevsky, Whitehouse and Staple, 1960; Whitehouse, Staple and Kritchevsky, 1960).

The effect of different types of fat in the diet has not been shown conclusively. Apparently conflicting results have been obtained by different workers. Lewis (1958) reported that the feeding of unsaturated fat to man resulted in increased excretion of bile acids. But Kritchevsky, Kolman, Whitehouse, Cottrell and Staple (1959) found that rat liver mitochondria from animals fed unsaturated fat oxidized cholesterol to a lesser extent than did mitochondria from rats fed a more saturated diet.

Control of Cholesterol Levels

Cholesterol synthesis is probably a continuous process in many organs, and in order to maintain a fairly constant blood level, the rate of synthesis must equal the rate of removal of cholesterol. The processes must be under very fine control as varying amounts of cholesterol are entering from the gut at

different times. In the preceding paragraphs are described factors which affect the cholesterol level in some way, but most of these factors are so miscellaneous that at present it is not possible to fit them into a coherent scheme.

The greatest bulk of quantitative work has been performed on plasma cholesterol, partly because of the ready availability of blood, and partly because the rapid turnover of plasma cholesterol renders it sensitive to changes in metabolism of other tissues and it may act as mirror to such changes. Labelling experiments have shown that there is rapid equilibration between the plasma and liver pools of cholesterol so that for many purposes they may be considered as a single pool (Eckles, Taylor, Campbell and Gould, 1955). Hence all the factors which affect hepatic biosynthesis and catabolism of cholesterol will probably also affect the blood level. A further complication is that the sterol may occur in different chemical forms. Since the molecule contains a hydroxyl group it can form esters at the 3-position. The esters formed physiologically are those with long chain aliphatic acids in the C_{12} to C_{24} range. The cholesterol present in the blood then can

be separated, not only into non-esterified and esterified fractions, but the latter can be sub-divided into many sub-fractions depending upon the chain length and degree of unsaturation of the fatty acids. Considering that each of these esters may suffer a different metabolic fate, this makes the concept of plasma cholesterol as a single entity rather unsatisfactory. However, at the present time, separation of the individual esters without prior hydrolysis is not a practical possibility, so until different techniques have been developed, probably in the field of gas phase chromatography, the reactions of individual esters cannot be followed.

Outline of the Work to be Described in this Thesis

Two aspects of cholesterol metabolism were chosen for further study; both were concerned with the degradation of cholesterol. One was the effect of dietary linoleic acid on the turnover of serum cholesterol in the rat. Up to the time that this work was started (1958) all the published studies on the effect of unsaturated fat on cholesterol metabolism were

concerned only with measurements of cholesterol concentrations. It was decided that an assessment of the circulating life of cholesterol under different dietary conditions might help to throw light upon the hypocholesteraemic effect of unsaturated fat, particularly to indicate whether linoleate might act by accelerating the degradation of cholesterol, or whether cholesterol biosynthesis might be decreased. The work consisted of measurements of the serum half-life of cholesterol synthesized from ^{14}C-labelled acetate by rats pre-fed diets containing different types of fat, and is described in Section 1 of this Thesis.

The second aspect of cholesterol metabolism which was studied was the chemical attack on the molecule, which is involved in the transformation to bile acids. This work falls into three parts, which form the subject matter of Section 2.

In Section 2A is described a search of various tissues for new oxidized derivatives of cholesterol. The isolation and identification of such a derivative was achieved.

The chemical synthesis of the material, together with its properties, is described in Section 2B.

In Section 2C, accounts are given of enzymic experiments which were designed to try to define the biochemical role of the isolated material

SECTION 1

THE EFFECT OF DIETARY LINOLEIC ACID ON THE TURNOVER OF SERUM CHOLESTEROL IN THE RAT

INTRODUCTION

Whilst all mammals are able to synthesize saturated long chain fatty acids, there appear to be species differences in the extent to which unsaturated acids of similar chain lengths can be produced.

The unsaturated fatty acids can be grouped in 'families' whose members may differ in chain length and total unsaturation, but resemble each other in the position of the double bond nearest to the terminal methyl group. The oleic acid family has the first double bond at the ninth carbon atom, whereas the linoleic acid family is first unsaturated at the sixth carbon atom and possesses two or more methylene-interrupted cis double bonds. Linoleic acid itself is 9:12-octadecadienoic acid (see Figure 3).

Arachidonic acid (5:8:11:14-eicosatetraenoic acid) is a member of the linoleic acid family, but linolenic acid (9:12:15-octadecatrienoic acid) forms the basis of a different family.

Linoleic acid was shown to be an essential growth factor for the rat by Burr and Burr in 1929. Deficiency of the acid also causes the

FIGURE 3

FORMULAE OF SOME POLYUNSATURATED FATTY ACIDS

$CH_3(CH_2)_4.CH{:}CH.CH_2.CH{:}CH.(CH_2)_7.COOH$

Linoleic acid

$CH_3.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH{:}CH.(CH_2)_7.COOH$

Linolenic acid

$CH_3.(CH_2)_4.CH{:}CH.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH{:}CH.CH_2.CH_2.CH_2.COOH$

Arachidonic acid

increase in the proportion of cholesterol which is esterified with unsaturated fatty acids.

Apparently contradictory results have been obtained by other workers (Klein, 1958; Swell, Flick, Field and Treadwell, 1955). However, the experimental conditions were very different. Klein fed his rats with linoleic acid to the extent of 30% by weight of the diet - which is approximately 55% of the caloric value. Experiments performed using so unbalanced a diet can hardly be interpreted as physiological. Klein claims that the rise in serum cholesterol is specifically related to the presence of the linoleic acid rather than to the high fat level, since the substitution of an equivalent amount of saturated fat did not elevate the cholesterol level to the same extent. It seems possible that the absorption of fat from the diet will be affected when the fat intake is so high, and that saturated and unsaturated fats may be absorbed at different rates. The diets of Swell contained taurocholic acid which would also have a marked effect on the absorption of the fat.

In contrast, the diets used by Hauge and Nicolaysen consisted of fat only to the extent of 10% by weight. This was usually saturated fat.

The animals were pre-fed this diet for several weeks and then the effect of a supplement of 50-80 mg. of unsaturated fatty acids per rat per day was observed. This is equivalent to about 1% by weight of the diet. It is obvious from only a brief consideration of the experimental conditions that the apparent discrepancies in the results obtained by different workers are to be expected since in all likelihood quite different phenomena have been observed.

In the work to be described in the Experimental Section, the diets were chosen to provide what is generally accepted to be a normal level of fat - 10 to 15% of the calories. It was observed however that control animals on a stock low fat diet which contained only 2% by weight of total lipid grew normally and exhibited no obvious deficiency symptoms.

When the cholesterol lowering effect of unsaturated fatty acids was first described, one explanation proposed was that there might be inhibition of cholesterol synthesis by the fatty acid. An alternative theory was that the unsaturated fatty acid might, in some way, increase the rate of breakdown of cholesterol to bile acids (Kinsell, Michaels, Friskey and Splitter, 1959).

The following work was undertaken in order to investigate the effect of a dietary supplement of unsaturated fatty acids upon the half-life of cholesterol in the rat.

GENERAL OUTLINE OF THE EXPERIMENTS PERFORMED

Young male rats were maintained on a stock low-fat diet for two to three weeks and then divided into groups which received different diets. A control group continued to receive the stock diet but test groups were given diets in which either 10 or 15% of the calories had been replaced by fat. In experiments I and IV the effect of pure methyl linoleate was compared with that of methyl stearate. In others (II, III, V and VI), commercially available food fats were used whose fatty acid compositions were known.

After a standard period on the experimental diets, usually three weeks, each rat was injected with a dose of sodium 1-^{14}C-acetate. Small blood samples were removed from their tails at intervals over the next few days. The specific activity of the serum cholesterol was measured, hence plots could be drawn representing the rate

of removal of labelled cholesterol from the serum. These were used to calculate the half-life of the serum cholesterol.

The experiment was performed six times over a period of two years. In experiments I and II the number of animals used was three per group; in III and IV, four per group; and in V and VI, eight. In experiments I and II, sera from the animals were pooled for each group; in the remaining experiments, sera were pooled for pairs of animals. It was not considered feasible to attempt estimations of specific activity on the serum of a single animal because it was desirable to take as small a blood sample as possible. This meant that the amount of cholesterol contained in a single sample was too small to be measured with the desired degree of accuracy. Pooling was also necessary in order to obtain a level of counting of the isotope which would be well above background, especially in the final samples where the activity was relatively low. Larger numbers of animals were not used because of the laborious nature of the determinations, and also because the cost of the ^{14}C-labelled acetate was a limiting factor.

Due to circumstances beyond the control of the investigator, the results obtained for experiment V were abnormal, and so cannot be included with the results from the other experiments; they will however be considered separately since they reveal some interesting facts. Consequently, only in experiment VI could the results be subjected to statistical analysis.

EXPERIMENTAL

Animals

White male inbred rats of the Wistar strain were used, their ages ranging from 54 to 77 days, and weights from 180 to 260 g. In any one experiment the animals used were litter mates as far as possible and were well matched for weight.

Diets

The composition of the stock low-fat diet was as follows:-

wholewheat flour	70%	
skimmed milk powder	25%	
dried yeast powder	5%	by weight.

The total fat content of this diet was 2% by weight and the sterol content (digitonin precipitable, Liebermann-Burchard positive material) was 0.05%. This would be due to plant sterols which are absorbed to a very small extent (Gould, 1955).

The experimental diets were made up by the replacement of part of the flour by lipid, so that caloric equivalence was retained. The lipids employed were:-

methyl linoleate
methyl stearate
sunflower seed oil
safflower seed oil
lard

Note: The methyl esters were used as methyl linoleate is more stable than is the free acid. The methanol which would result on biochemical hydrolysis would be in too low concentration for toxicity to be a problem. Methyl stearate was used however to account for any complications which might arise through the use of the esters.

TABLE 1

Expt. No.	Diet	Duration of Diet (days)
I	a) Stock b) + 10% Methyl linoleate c) + 10% Methyl stearate	8
II	a) Stock b) + 10% Sunflower seed oil	17
III	a) Stock b) + 15% Sunflower seed oil	14
IV	a) Stock b) + 10% Methyl linoleate c) + 10% Methyl stearate	7
V	a) Stock b) + 10% Safflower oil c) + 10% Lard	14
VI	a) Stock b) + 10% Safflower oil c) + 10% Lard	19

Details of the sources of the dietary components will be found in the Appendix Section.

Table 1 shows a summary of the conditions for each experiment including the type of lipid used and its level in the diet.

The mass of diet fed was approximately 10% of the animals' body weight. After they had been fed with the stock diet for a given period (see Table 1), the rats were divided into experimental groups and were given the different diets. The diets were made up freshly twice a day so that decomposition of the unsaturated fatty acids would be minimized.

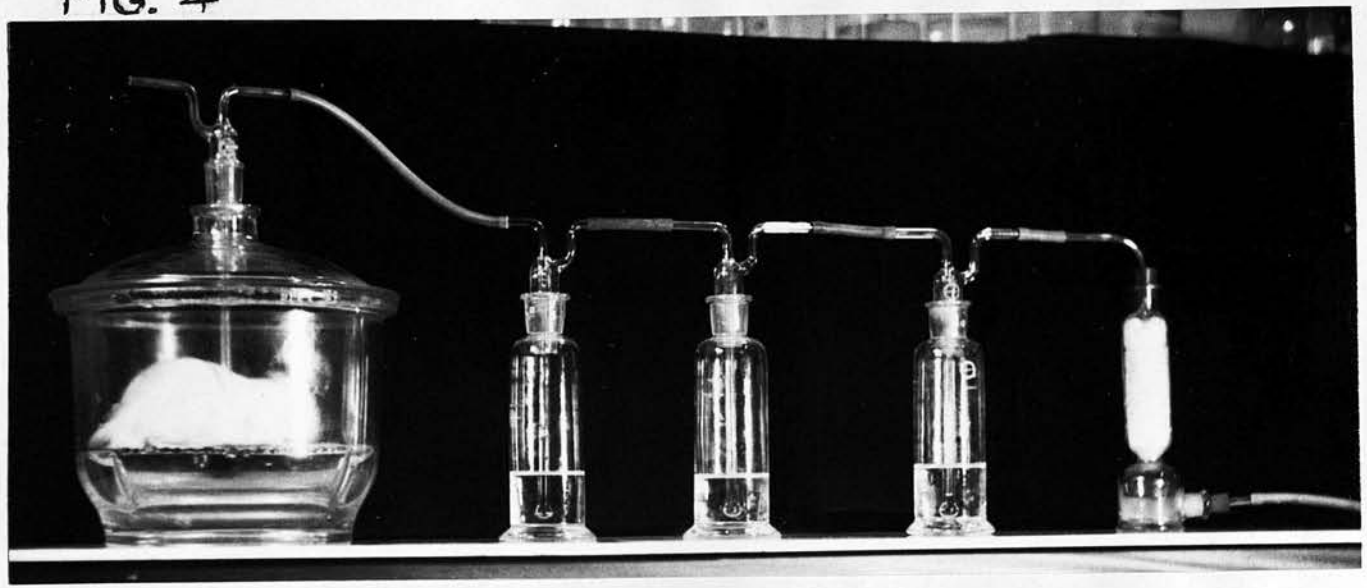
The animals had free access to water.

All the diets used were capable of supporting normal growth and no significant differences in weight were observed between animals on different diets.

Administration of ^{14}C-Labelled Acetate (acetate-1-^{14}C)

The dose given was 30 μc. per rat in experiments I-IV, and 50 μc. in experiments V and VI. This was present as 100 μmoles in 0.2 ml. The solution was injected into the rat intraperitoneally.

FIG. 4



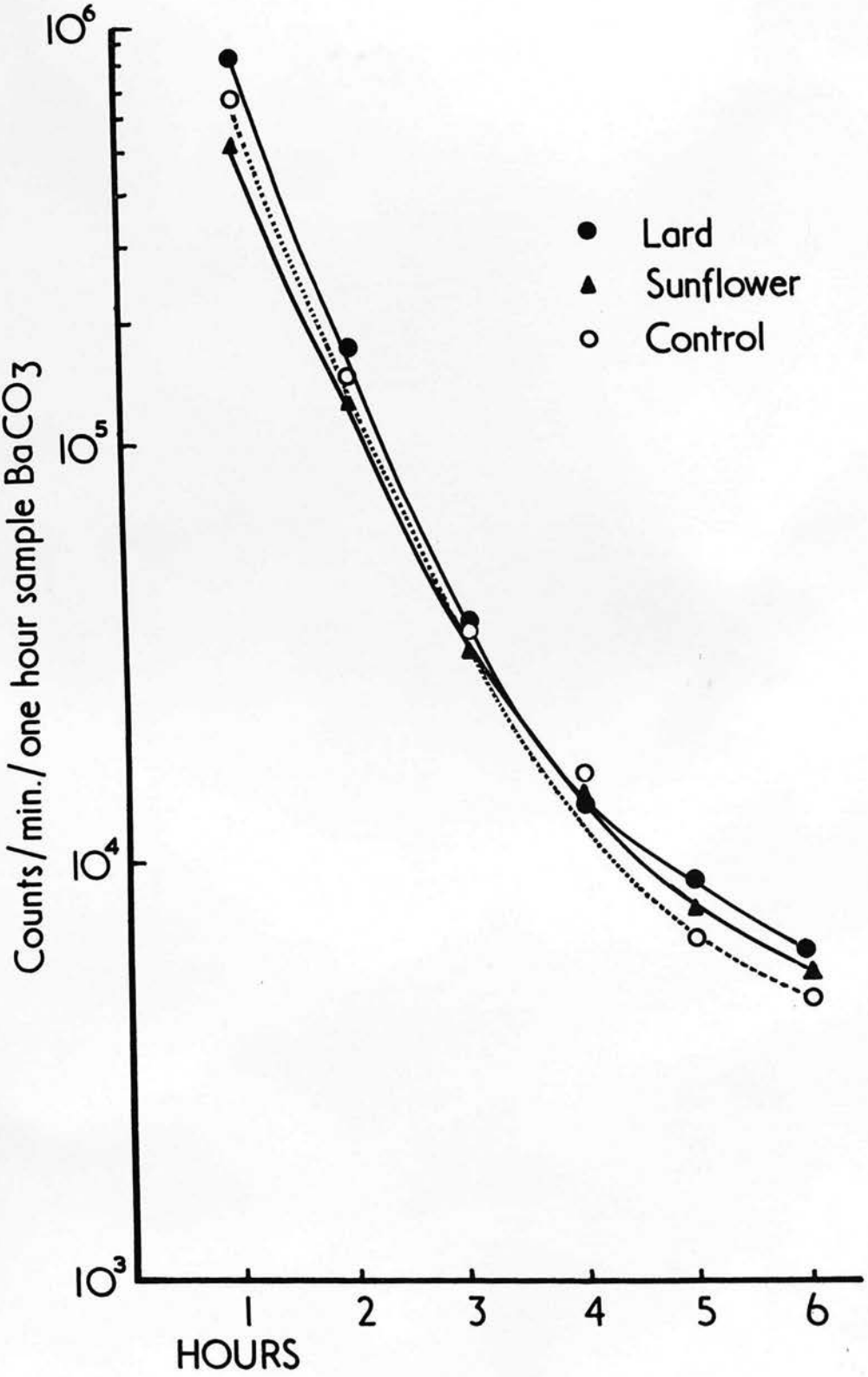
Collection of Blood Samples

Blood was withdrawn from the tail by cutting off a small piece with a sharp scalpel blade, and gently 'milking' blood from the cut end into a specimen tube. The method is described in detail by McGuire (1956). By means of this method, 0.5 ml. of blood could be collected from the animal without difficulty and without causing the rat undue stress. The times at which blood samples were withdrawn differed slightly with each experiment, and are indicated, together with the analytical results, in the Appendix.

Procedure

After a given period on the experimental diets (see Table 1), the animals were injected with the labelled acetate. For at least six hours after the injection the animals were kept in closed circuit metabolism cages, and the expired carbon dioxide was trapped in potassium hydroxide solution. A diagram of the apparatus is shown in Figure 4. The efficiency of the apparatus depended upon the water-pump which was responsible for drawing an adequate stream of air through the cages. (The loss of experiment

FIG. 5


10^6
10^5
10^4
10^3
Counts/min./one hour sample $BaCO_3$
Lard
Sunflower
Control
1
2
3
4
5
6
HOURS

V which has already been mentioned was due to a sudden failure in the water pressure. The short period of anoxia to which the animals in this experiment were subjected, resulted in the death of a number of them, and a marked impairment of metabolism of the survivors.)

The expired CO_2 samples were collected hourly and precipitated as barium carbonate. The precipitates were filtered on to a plate and counted as a film at infinite thickness using an end-window counter. Details of the procedure are given in the Appendix.

The results of the counting were plotted against time. A typical set of curves is seen in Figure 5.

Treatment of Blood Samples

The samples were allowed to clot and were then centrifuged to free the serum. From each sample 0.2 ml. was removed, using a blood pipette, and these were pooled according to the number of animals in the group. The sera were extracted by refluxing with acetone-alcohol (1:1), cooling and centrifuging, repeating the procedure and pooling the extracts. These were

then hydrolysed so as to estimate total cholesterol (i.e. the sum of esterified plus unesterified). Part of each extract was used for cholesterol estimation and part for determination of radioactivity. In each case the method involved a digitonide precipitation after the manner of Sperry and Webb (1950). The details of these procedures are given in the Appendix.

The specific activity of the serum cholesterol was thus determined and the log of this value was plotted against time for each group of animals.

RESULTS

The plots were used to determine the serum half-life of cholesterol for each group (Figures 6, 7, 8 and 9). During the period of observation the plots were almost linear and could be extrapolated back to zero time, but after 100 hours the curves appeared to flatten out (see Figure 7). The point at which the extrapolated plot intersected the ordinate can be regarded mathematically as representing the incorporation of isotope into cholesterol at zero time. However, to regard this as an index of

FIG. 6

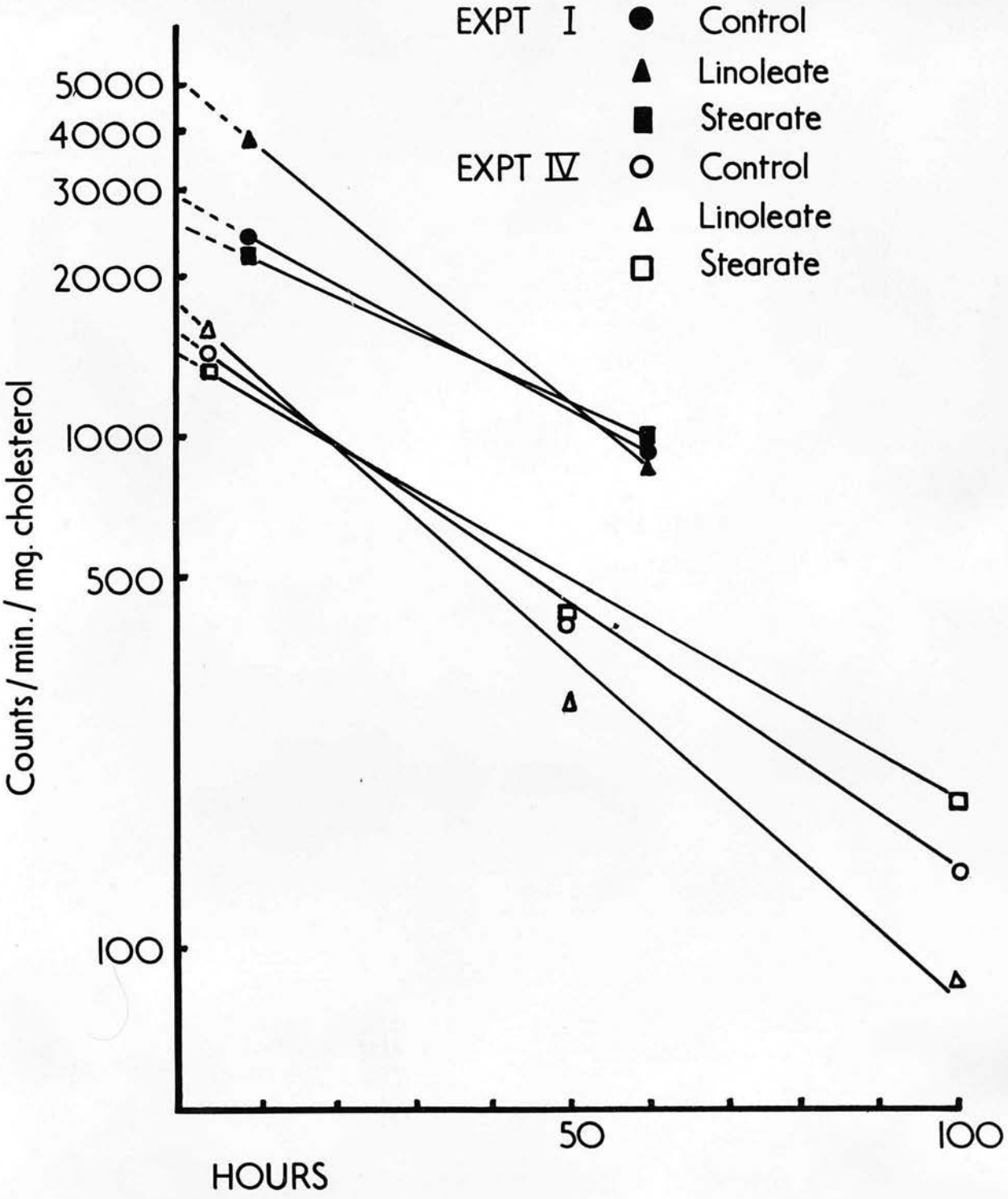

EXPT I
Control
Linoleate
Stearate
EXPT IV
Control
Linoleate
Stearate
5000
4000
3000
2000
1000
500
100
Counts / min. / mg. cholesterol
50
100
HOURS

FIG. 7

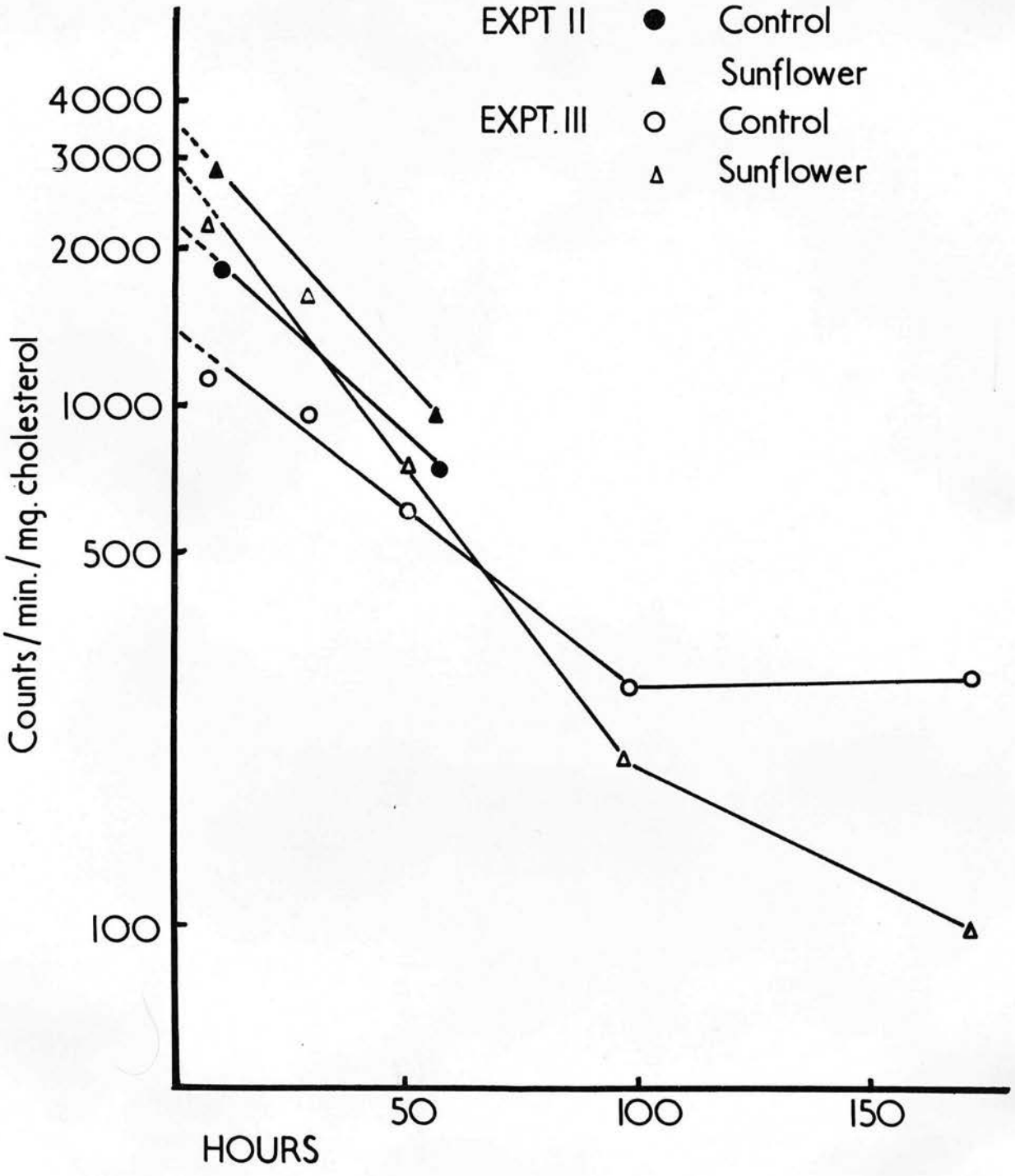

EXPT II
Control
Sunflower
EXPT. III
Control
Sunflower
4000
3000
2000
1000
500
100
Counts/min./mg. cholesterol
50
100
150
HOURS

FIG. 8

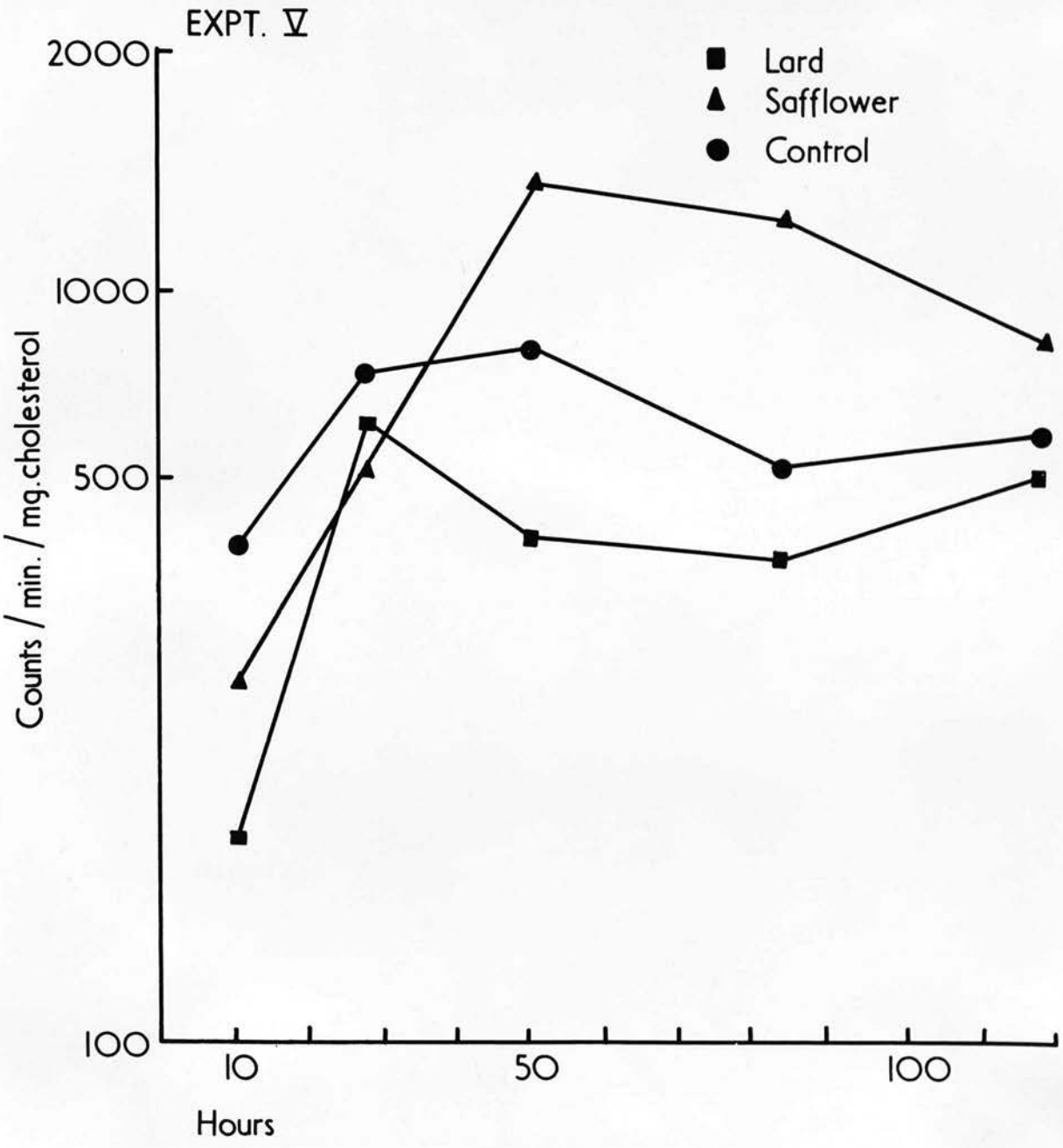
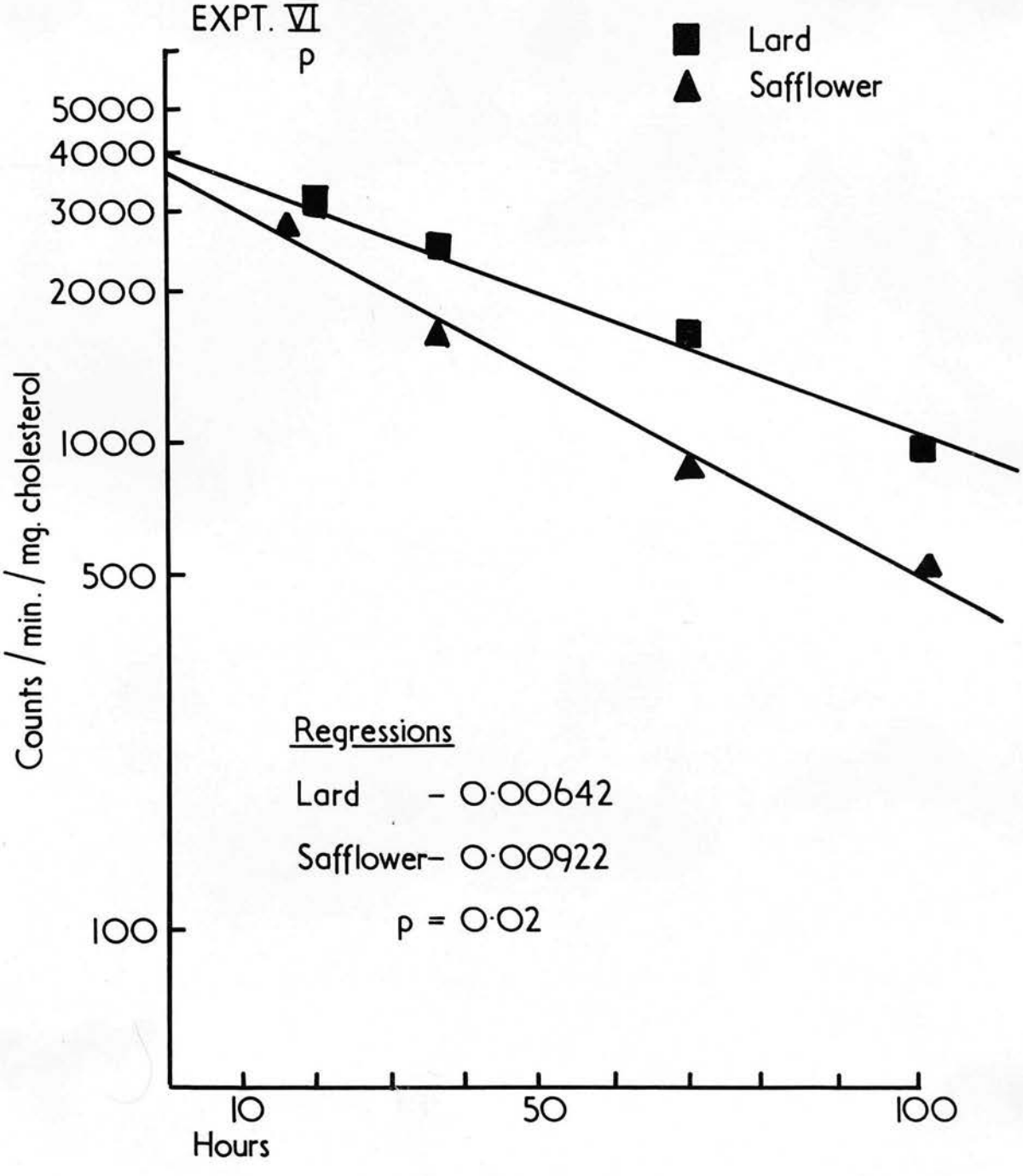

EXPT. V
2000
1000
500
100
Counts / min. / mg. cholesterol
Lard
Safflower
Control
10
50
100
Hours

FIG. 9


EXPT. VI
P
Lard
Safflower
5000
4000
3000
2000
1000
500
100
Counts / min. / mg. cholesterol
Regressions
Lard – 0·00642
Safflower– 0·00922
p = 0·02
10
50
100
Hours

the rate of synthesis of cholesterol by the animals is only justifiable if certain assumptions hold good. This question will be considered in the Conclusions (p. 28). The results are summarized on p. 130. The 'relative rate of incorporation of isotope' is measured as the percentage incorporation of ^{14}C into cholesterol at zero time for each experimental group, using the control group as a 100% standard. The feeding of methyl linoleate and of sunflower seed oil resulted in a marked decrease in the 'half-life' of serum cholesterol, whereas feeding methyl stearate or lard resulted in an elongation of the half-life. In experiment VI, where statistical analysis was possible, the slopes of the lines for the experimental groups were significantly different. The results of the 'relative rate of incorporation' were rather less consistent. The initial incorporation of ^{14}C was greater for the methyl linoleate and sunflower seed oil groups, and less for those fed on stearate. In experiment VI, all the extrapolated values fell close together and that for the lard group was slightly higher than that for either the control or safflower seed oil groups.

Results of Experiment V

See Figure 8.

Despite the higher dose of acetate used in this experiment (50 μc. instead of 30 μc. per rat) the activity of serum cholesterol after 10 hours is lower than normal by a factor of about 8. The activity then rises, reaching a maximum around 50 hours for the safflower and control groups and between 27 and 53 hours for the lard group. After the maximum the safflower group decrease fairly sharply, but the control and lard groups fluctuate a little but show no significant trend. The very low initial incorporation would suggest that virtually all hepatic synthesis of cholesterol was suppressed by the period of anoxia, since the liver and blood pools equilibrate very rapidly. This conclusion is supported by the fact that livers removed from the animals which died contained virtually no labelled cholesterol at a time, approximately 1 hour after injection, when incorporation of acetate into cholesterol should have been high.

The rise in activity to a level which was approximately half the normal by 50 hours would indicate that labelled cholesterol was entering

the plasma after synthesis in other organs. Some of this would be hepatic in origin, but presumably contributions would also be made by skin, kidneys, adrenals and gut.

The entry of this labelled cholesterol into the plasma pool, at a time when the activity of the liver/plasma cholesterol is declining, may help to account for the slight departure from linearity observed in the normal experiments.

CONCLUSIONS

These experiments were designed to study the effect of small amounts of unsaturated fatty acids on sterol metabolism. The basic diet employed was a low fat one which nevertheless was capable of supporting the normal growth of young male rats. No signs of 'essential fatty acid' deficiency were observed in any of the animals maintained on this regimen. The lipid was added to this diet at a level to replace 10% or 15% of the calories, and this percentage of dietary fat is considered to be well within a physiological range. The results obtained in this study were comparable when either pure linoleic acid or

oils rich in linoleate were used. When these diets were fed to the rats the turnover of serum cholesterol was increased compared with either low fat control animals or with animals on a saturated fat diet.

From the results obtained in this study, extrapolation of the specific activity/time curves suggests that the percentage incorporation of isotope into cholesterol is usually increased in those animals pre-fed a diet containing poly-ethenoid fatty acids. This result is only of significance, however, if the tracer dose of acetate were delivered into 'acetate pools' of comparable size for each group. It was considered likely that this was the position since all the animals were of similar weight and had been fed isocaloric diets. The specific activities of the expired carbon dioxide in all groups were closely similar, which suggested that the acetate pools were of comparable size, but, as such a large proportion of the acetate is converted to CO_2 compared with that converted to cholesterol it is possible that small fluctuations in the acetate pool size might

affect cholesterol metabolism without causing any marked change in the pattern of CO_2 excretion. If the acetate pool sizes of the various groups are similar, one interpretation of the increased relative percentage incorporation of isotope into cholesterol by the unsaturated fatty acid (UFA) fed animals is that these animals showed an increased rate of sterol synthesis in most cases by comparison with those on a saturated fat diet. The apparent discrepancy in the results of experiment VI may be accounted for by the fact that the saturated fat used was lard instead of the pure methyl stearate used in the earlier experiments. It is conceivable that the lard may have contained sufficient UFA to be effective at the levels fed (the iodine no. was 65). More conclusive results might have been obtained in this experiment had a fully hydrogenated fat been used. In this experiment though, the usual effect on the serum cholesterol half-life was still observed.

The specific activity/time curves (Figures 6-9) suggest that while probably more cholesterol is being synthesized by the animals pre-fed the unsaturated diet, there is also an increased rate of removal or catabolism of

serum cholesterol. It would seem, therefore, that the UFA may affect sterol metabolism by at least two mechanisms which may or may not be related. Since there is no evidence that cholesterol is removed from the blood to be passively stored elsewhere in the body, the only simple explanation would be that the UFA exerts an influence on cholesterol degradation.

Since this study was commenced in 1958, work has been reported in the literature which leads to similar conclusions.

Avigan and Steinberg (1958) reported that rats pre-fed on corn oil - another oil rich in linoleic acid, exhibited an increased incorporation of ^{14}C into liver cholesterol when injected with labelled acetate shortly before they were killed. Merrill (1958) observed a similar result when using linoleic acid. Wood and Migicovsky (1958) also observed an increased incorporation of labelled acetate into liver cholesterol in rats fed unsaturated diets both *in vivo* and *in vitro*. In the latter case, these workers measured the incorporation of radioactive acetate into cholesterol by homogenates of livers from UFA fed animals. The same technique was used and similar results reported by

Mukherjee and Alfin-Slater in 1958. Since liver and blood cholesterol equilibrate with each other so rapidly, they are usually regarded as forming a single pool and hence these results obtained for liver cholesterol seem to be complementary to the observations on serum cholesterol described in this study.

The mechanism for this effect on cholesterol synthesis is obscure. It is possible that the UFA might affect cholesterol synthesis indirectly by some permeability or transport mechanism, perhaps controlling the rate of movement of intermediates from one part of the cell to another; or more directly by combining with already formed cholesterol to form esters. If these UFA esters were more readily metabolized than either free cholesterol or its saturated esters, then the equilibrium between free and ester cholesterol in the liver would be disturbed. This might well be a factor governing the rate of biosynthesis of cholesterol. The second conclusion drawn from this study, namely that cholesterol turnover is increased in the presence of unsaturated fatty acids, would support the above hypothesis. It seems reasonable to suggest that the UFA exert their

effect by the formation of specific cholesterol esters which are more metabolically active. Work has been reported by Klein and Martin (1959) in which the turnover of individual cholesterol esters in liver was measured. They found that the turnover of unsaturated esters was very much greater than that of the saturated ones.

These ideas will be considered in more detail in the Discussion (p. 105) where attempts are made to fit into a more general scheme of cholesterol metabolism the observations described in this Section.

SECTION 2

STUDIES ON DERIVATIVES OF 7-HYDROXYCHOLESTEROL

INTRODUCTION

The pioneer of studies of cholesterol oxidation was Lifschütz. As long ago as 1914 he suggested that the compound produced by air oxidation of cholesterol which he designated 'oxycholesterol' may also have been a physiological breakdown product of cholesterol (Lifschütz, 1914).

'Oxycholesterol' was subsequently shown to be a complex mixture consisting predominantly of 7-keto-cholesterol (cholest-5-en-3β-ol-7-one) and the epimeric 7-hydroxycholesterols (cholest-5-en-3β:7α-diol) (Bergstrom and Wintersteiner, 1941). The similarity of a colour reaction to ferric chloride given by both 'oxycholesterol' and the bile acids led Lifschütz to postulate that there may be a metabolic relationship between cholesterol and bile acids.

During the period 1910 to 1930 the chemical structures of both cholesterol and bile acids were elucidated (Windaus, 1932; Wieland and Dane, 1932), and the close similarity between them made the earlier hypothesis seem likely, but no investigations were able to show a physiological relationship until the work of Bloch and his coworkers in 1943 (Bloch, Berg

and Rittenberg, 1943). They fed deuterated cholesterol to a dog with cholecystonephrostomy and were able to isolate labelled cholic acid from the urine.

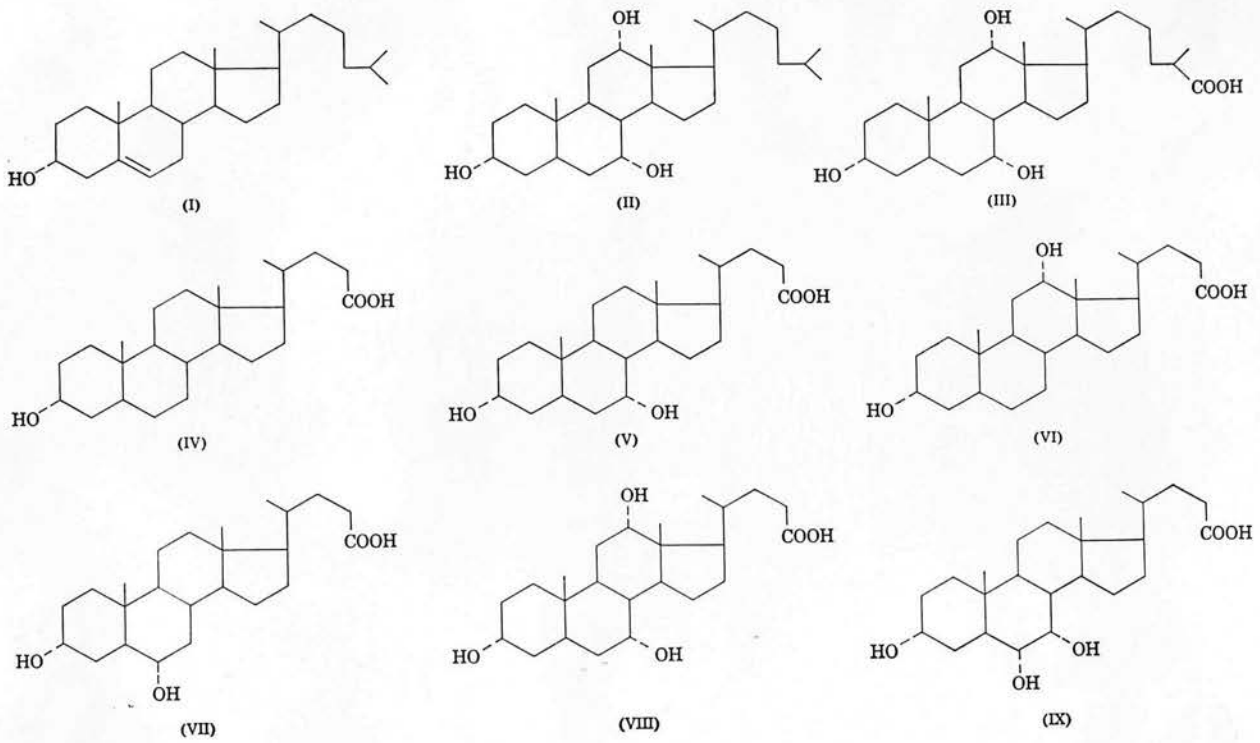
When ^{14}C labelled cholesterol became available in 1950, the quantitative aspects of bile acid formation could be studied. Siperstein and Chaikoff (1952) found that 80 to 90% of a dose of ^{14}C-cholesterol administered to rats was excreted in faeces as acidic products in fifteen days, the remainder being excreted as unchanged cholesterol and as neutral sterols formed from cholesterol by intestinal bacteria. Similar results have been obtained for other mammals, e.g. man and rabbit (Rosenfeld, Hellman and Gallagher, 1955; Siperstein and Murray, 1955; Ekdahl and Sjovall, 1955).

The modifications which require to be made in the conversion of cholesterol to cholic acid (3α:7α:12α-trihydroxycholanic acid) are as follows:-

1. Inversion of the 3β-hydroxyl group and saturation of the $\triangle^5$ to the AB cis configuration.

2. Introduction of the hydroxyl groups at the 7 and 12α-positions.

FIG. 10



(I) cholesterol. (II) 3α,7α,12α-trihydroxycoprostane. (III) 3α,7α,12α-trihydroxycoprostanic acid. (IV) lithocholic acid. (V) chenodeoxycholic acid. (VI) deoxycholic acid. (VII) hyodeoxycholic acid. (VIII) cholic acid. (IX) hyocholic acid.

3. Degradation of the side chain with the formation of a carboxyl group at C_{24}.

The order of the transformations and many of the details are still not fully understood. (The formulae of cholic acid and the other commonly occurring bile acids are shown in Figure 10.)

Work on the removal of the sidechain has led to the conclusion that cholesterol derivatives with hydroxyl groups at the 25 and 26 positions, or with a double bond between carbons 25 and 26, are compounds formed prior to the splitting of the sidechain (Lynn, Staple and Gurin, 1955; Fredrickson and Ono, 1956).

The majority of the work on cholesterol degradation has been performed by the Swedish School of Bergstrom and his coworkers who have used the bile fistula rat to study the metabolism of hypothetical intermediates. These workers have also performed <u>in vitro</u> studies with possible intermediates using rat and mouse liver preparations.

By means of these techniques they have been able to show that modification of the nucleus precedes the removal of the sidechain. The experiments were as follows:-

3β-Hydroxy: Δ^5-cholenic acid and coprostane-3α:7α:12α-triol were both prepared labelled with ^{14}C and administered to bile fistula rats; whilst the latter compound was readily converted to cholic acid, the former was excreted in the form of an unidentified acid (Bergstrom, Paabo and Rumpf, 1954; Bergstrom, 1955).

These results indicated that substitutions of the nucleus to form cholic acid do not occur after oxidation of the sidechain.

In a similar way information has been obtained with regard to the stage at which inversion of the C_3 hydroxyl group and saturation of the Δ^5 double bond occur. If these changes preceded the hydroxylations of the nucleus then the following compounds might have been precursors of cholic acid:-

Cholest-5-en-3α-ol, coprostan-3β-ol, coprostan-3α-ol, cholest-4-en-3-one, cholest-5-en-3-one.

None of these compounds was converted to cholic acid in the bile fistula rat (Bergstrom, 1955; Harold, Chapman and Chaikoff, 1957; Harold, Jayko and Chaikoff, 1955). It seems likely, therefore, that the hydroxylation of the nucleus is one of the earliest stages in bile

acid formation.

Since the work to be described in this Section of the Thesis is concerned with the early stages in the degradation of cholesterol, work performed on the later stages will not be covered here. Details of this and other aspects of bile acid formation are reviewed by Bergstrom, Danielsson and Samuelsson (1960).

The bile acid which has been considered so far is cholic acid, which is regarded as the parent, as it forms about 80% of the total bile acids in the rat. Most of the remainder is chenodeoxycholic acid (3α:7α-dihydroxycholanic acid) (Bergstrom and Sjövall, 1954).

Two other trihydroxy acids (3α:6β:7α-trihydroxycholanic acid and 3α:6β:7β-trihydroxycholanic acid) are formed to a minor extent (Hsia, Matschiner, Mahowald, Elliott, Doisy, Thayer and Doisy, 1957, 1958).

The rat is unique in that the liver contains an enzyme which can insert a 7α-hydroxyl group into deoxycholic acid (3α:12α-dihydroxycholanic acid) (Bergstrom, Rottenberg and Sjövall, 1953). This is a curious fact, since the deoxycholic acid itself is not a product of the rat's metabolism, but is formed from cholic

acid by intestinal flora and is then absorbed and enters the entero-hepatic circulation (Norman and Sjövall, 1958).

The conjugation of the bile acids with glycine or taurine to form bile salts has been shown to occur in liver microsomes (Bremer and Gloor, 1955), and so only the free acids are isolated when in vitro work is performed using liver mitochondrial preparations.

While both 7α-hydroxycholesterol and coprostan-3α:7α-diol can be converted to cholic acid and chenodeoxycholic acid in the rat (Lindstedt, 1957; Bergstrom and Lindstedt, 1956), neither chenodeoxycholic acid nor 3α:7α-dihydroxycoprostanic acid can be thus converted. Hence modification of the sidechain renders 12-hydroxylation impossible.

It appears likely that hydroxylation at the 7 position is one of the earliest steps in cholesterol oxidation, and so Lifschütz's early speculation seems correct.

Despite the demonstration by Lindstedt (1957) that 7α-hydroxycholesterol could be converted to cholic acid, there has been no conclusive proof that cholesterol is converted to 7α-hydroxycholesterol in the liver, since it is difficult

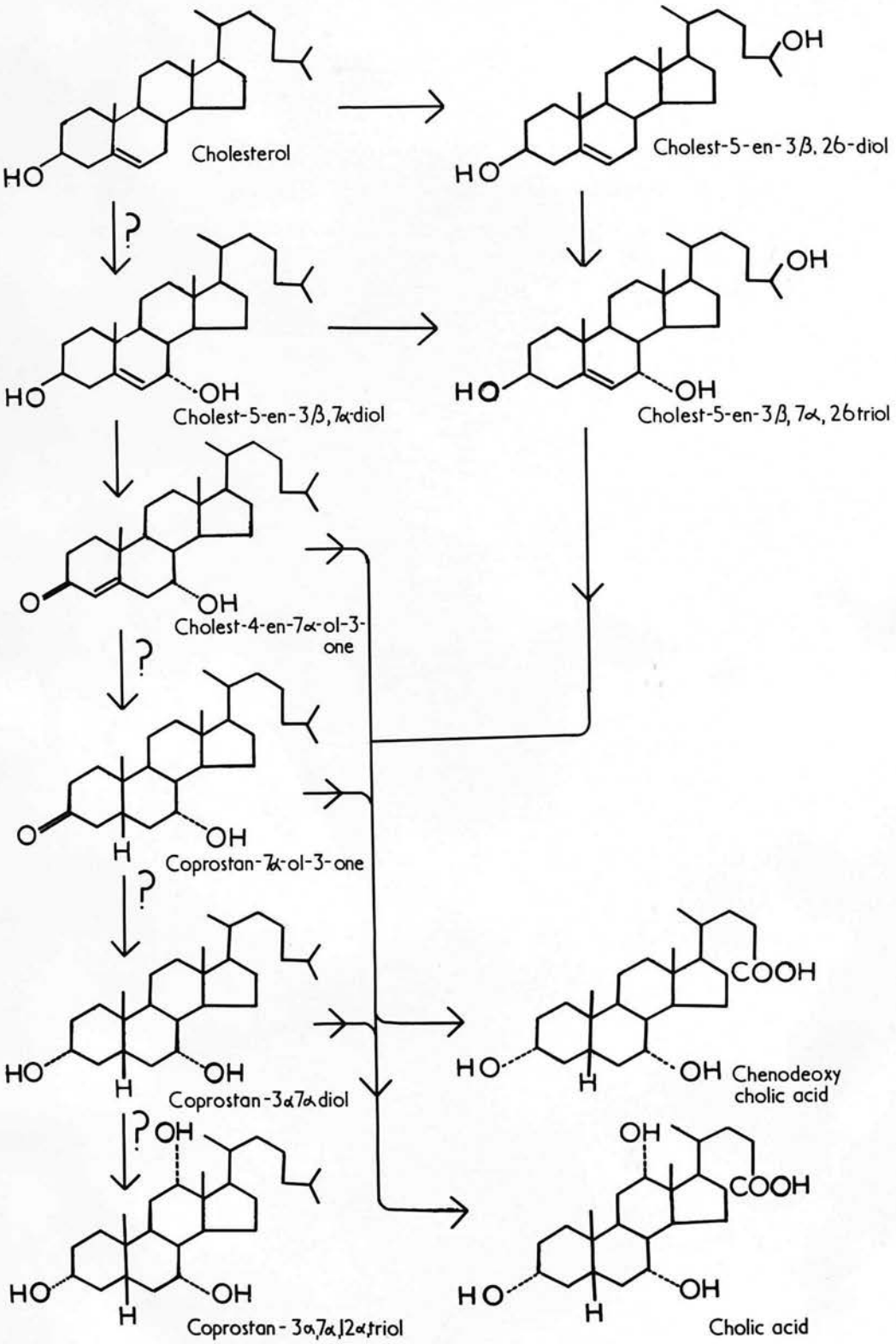
to exclude completely the effect of autoxidation. Nevertheless, a considerable amount of work has been done on the 7-hydroxylation step. Bergstrom believes that this might be the rate-controlling step for the whole sequence since 7-hydroxylated compounds are converted to bile acids more rapidly than is cholesterol (Bergstrom, 1958).

The steric course of the hydroxylation has been studied by Bergstrom, Lindstedt, Samuelsson, Carey and Gregoriou (1958) using cholesterol stereo-specifically labelled with tritium in position 7α or 7β. The cholic acid produced retained the tritium exclusively in the 7β-position, indicating that the 7α-hydroxylation had been direct and that no 7-keto intermediate was involved.

Samuelsson (1960) showed that the inversion of the 3β-hydroxyl group probably involves a ketone formation since cholesterol tritiated at the 3α-position loses the isotope on conversion to cholic acid. An interesting corollary to this was the discovery by Danielsson (1961a) that 7α-hydroxycholesterol could undergo reduction in the presence of DPN to a less polar compound which proved to be cholest-4-en-7α-ol-3-one.

FIG. 11

Possible Intermediates in the Formation of Bile Acids in the Rat



The presence of this compound had been suspected by Yamasaki, Noda and Shimizu (1959) but they failed to characterize the material.

Danielsson (1961b, c) then showed that both this compound and the corresponding saturated one, coprostan-7α-3-one, were rapidly converted to bile acids, but as yet he has not shown the conversion of cholesterol to these compounds.

Figure 11 shows a summary of present knowledge in bile acid formation.

From this brief survey of present knowledge of bile acid formation, it can be seen that the greatest lack of information is in the early stages of cholesterol degradation. The work which will be described in Section 2A was undertaken in order to look for possible early oxidation products of cholesterol. Since it was believed that 7-hydroxylation is an early step in the sequence (see p. 40), it was decided to analyse lipid extracts of various organs by means of paper chromatography to search for traces of possible oxidation products.

Because the danger of artifactual oxidation was known, care was taken to work under anaerobic conditions wherever possible. The details of this phenomenon are described on pp. 45 and 110.

Methods had to be developed for both the extraction and the chromatography, as existing ones were not suitable.

As a result of this investigation, small amounts of material which reacted in every way as a 3-acyl ester of 7α-hydroxycholesterol were isolated from rat plasma, skin and liver, and from human plasma and leucocytes. Section 2A consists of a description of the isolation and identification of this material. The identification was achieved by comparison with a series of esters, whose chemical synthesis and properties are described in Section 2B. In order to try to elucidate the biochemical role of the esters, preliminary enzymic experiments were performed which are described in Section 2C. These showed that the esters can be both formed and hydrolysed biochemically and that they can be converted to bile acids.

The possible significance of these esters and their place in cholesterol catabolism is considered in detail in the Discussion on p. 113.

SECTION 2A

ISOLATION AND IDENTIFICATION OF NEW LIFSCHUTZ CHROMOGENS

The work to be described was undertaken to search for new possible intermediates in cholesterol degradation.

EXPERIMENTAL

Lipid extracts of serum or plasma were screened in a paper chromatographic system for traces of cholesterol derivatives. The system used was that of Michaleč (1958) which involved two-dimensional chromatography of an alcohol-ether extract. The extraction procedure was altered later as ether was found to have a destructive effect on some of the compounds concerned, and the chromatographic technique was modified to give a less cumbersome one-dimensional method (see Appendix for details).

The colour reagent used to pick out the spots on paper was ethanolic phosphotungstic acid 10% (w/v) (Martin, 1957). Data concerning the specificity of the reagent were compiled,

using standard compounds of the type that might be expected to occur in the extracts. It was found that both free and esterified cholesterol gave a salmon pink colour, whereas derivatives containing a 7-hydroxyl group and retaining the 5-6 double bond gave an intense blue. Details of the compounds tested are given in the Appendix.

When the papers were sprayed with the reagent the expected spots were seen for cholesterol and cholesterol esters, but in some cases a spot was observed with a lower R_F and which stained blue. Since this indicated that the material probably contained a 7-oxygen function, further investigation was considered desirable.

Strict precautions were taken against oxidation in all the ensuing work since cholesterol is notoriously prone to the formation of oxidative artifacts, some of which are 7-oxy compounds (Bergstrom and Wintersteiner, 1941). Biological material was extracted as rapidly as possible; temperatures were kept low; evaporations were performed in vacuo or under nitrogen.

The paper system was changed to a one-dimensional one which was still reversed phase.

The system was characterized with standard compounds whose R_F's are shown in the Appendix.

The lipid extracts were further fractionated on columns in order to obtain a less complex fraction for paper chromatography. The system involved the use of silicic acid columns with a mobile phase of petroleum ether containing increasing concentrations of chloroform (see Appendix).

The cuts were concentrated individually and applied to paper. On spraying the subsequent chromatograms it was found that the blue material occurred in the same cuts as those containing cholesterol. The mobility of the new blue staining material (which will be referred to now as BSM), both on paper and on the silicic acid column, was inconsistent with the polarity of 7-hydroxycholesterol although giving the same colour reaction. It would, however, correspond well with 7-hydroxycholesterol in which one of the hydroxyl groups had become esterified with a longchain fatty acid.

Isolation of BSM

In order to isolate BSM it was necessary to separate it from the accompanying cholesterol and

this was done in two ways.

1. By elution of the material from the paper chromatogram.
2. By the use of a reversed phase column system modified from those described by Danielsson (1958).

The first method proved tedious since the chloroform used for the elution also removed some paraffin from the paper and the mixture had to be separated on a second silicic acid column. The second method utilized a column of celite impregnated with dichlorodimethyl-silane and a partition system of chloroform/methanol/isopropanol/water. Using this type of column, separation was achieved (see Appendix for details).

BSM was detected in the cuts since it gave a positive Lifschütz reaction as well as a Liebermann-Burchard test (Liebermann, 1885; Burchard, 1889; Lifschütz, 1908; Bergstrom and Wintersteiner, 1942). The Lifschütz reagent gives a bright turquoise colour (λ max. 640 mμ) with 7-hydroxycholesterol and related compounds (see Appendix), but not with cholesterol. Concentration of the Lifschütz-positive cuts gave an extract which on paper ran as a single blue-

TABLE 2

SOURCES OF BSM

Species	Conditions		Tissue	Result
Rat	normal	male	serum	+
	normal	male	serum	+
	normal	female	serum	+
	fed thio-uracil	male	serum	+
	normal	male	serum	-
	normal	male	liver	-
	normal	male	spleen	-
	normal	male	kidney	-
	normal	male	skin	+
	normal	female	skin	+
	normal	female	liver	+
	fed saf-flower oil	male	liver	+
Man	normal	pooled	serum	+
	normal	pooled	serum	-
	female patient with haemochromo-tosis		haemo-lysed serum	-
	coronary patients	pooled	haemo-lysed serum	+
	leuco-cytes	pooled	leuco-cytes	+
same group throughout, pooled	patients prefed safflower oil		serum	+
	patients on low-fat diet following safflower oil		serum	+
	patients on saturated fat diet		serum	-
same subject	normal female control		serum	-
	21 days on saf-flower oil		serum	+
	21 days after stopping saf-flower oil		serum	-

staining spot with the same R_F as the corresponding spot in the original mixture. The total quantity of BSM obtained in this way was probably about 2 mg., but a substantial portion was taken for infrared analysis and was not recoverable, leaving only about 200 µg. for other identification purposes.

The average amount obtained from 20 ml. of human serum was about 200 µg. before purification. This corresponds to approximately 0.5% of the total cholesterol.

Identification of BSM

The total pooled samples from various sources (see Table 2) were used for identification purposes. The chromatographic behaviour of the material suggested that it might be 7-hydroxycholesterol esterified at one of the hydroxyl groups.

Hydrolysis of BSM

On mild alkaline hydrolysis with potassium carbonate at room temperature, a product was obtained which was chromatographically identical with 7-hydroxycholesterol. No other product was detectable.

TABLE 3

INFRARED ABSORPTION DATA

Compound	Abs. Band cm^{-1}	Inference
BSM Rat	1740) 1170)	long chain fatty acid ester
	1712) 1120) weak	traces of ketonic & ether impurities
BSM Human	1740) 1170)	long chain fatty acid ester
	1710	weak ketonic impurity
7α-Hydroxy-cholesterol	3600 + 1052	equatorial hydroxyl
	3600 + 1012	axial hydroxyl
7α-Hydroxy-cholesteryl myristate	3600 + 1010	axial hydroxyl
	1734 + 1174	long chain fatty acid ester
Cholesteryl linoleate	1732 + 1172	long chain fatty acid ester
	975	trans double bond

Note: The analyst commented that in the poor quality spectra of the BSM samples, it was unlikely that the absorption bands for a hydroxyl group would be evident, even if the group were present.

Infrared Absorption Analysis

A sample which was sent for infrared analysis was shown to be esterified at the 3-position, but no 7-hydroxyl group could be detected. The absorption pattern indicated the presence of a small amount of ketone impurity (see Table 3). It was obvious that the material had undergone some change during the procedures of purification and storage, since the originally isolated material was not a cholesterol ester as the infrared absorption might now indicate. The ability to give a positive Lifschütz reaction and a phosphotungstic acid (PTA) stain was lost during purification. The most obvious possibility was that the material as originally isolated was a 3-acyl ester of 7-hydroxycholesterol, and during isolation had become dehydrated.

This possibility was strengthened by the fact that on the paper system BSM did show slight traces of u.v. absorbing material of lower R_F, although the blue-staining spot was no longer visible. Unfortunately there was insufficient material left to perform a u.v. spectrum so there was no means of knowing the characteristics of this absorption.

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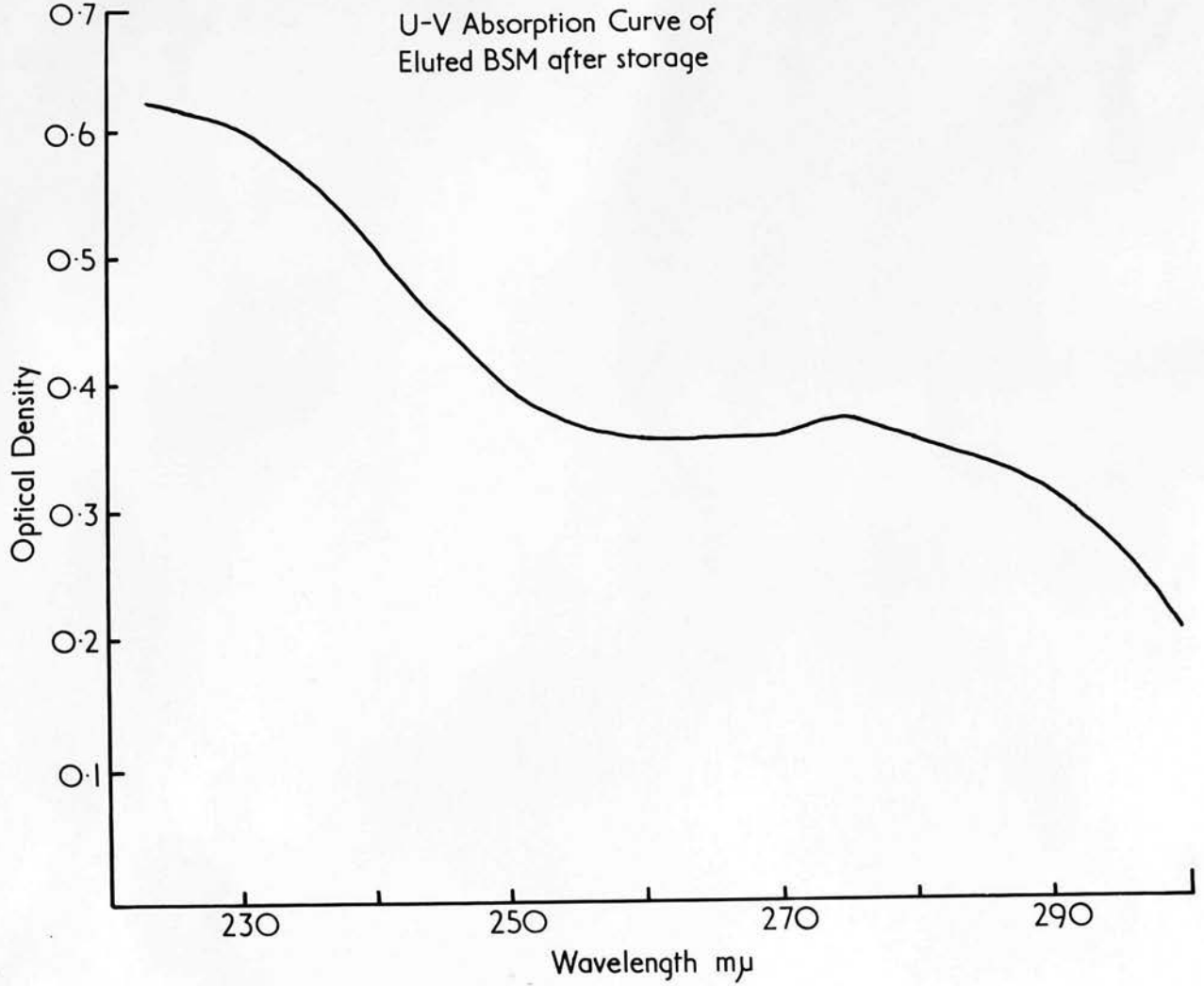
FIG. 12


U-V Absorption Curve of
Eluted BSM after storage
Optical Density
0·7
0·6
0·5
0·4
0·3
0·2
0·1
230
250
270
290
Wavelength mμ

Ultraviolet Absorption Analysis

However, a u.v. absorption spectrum had been obtained for the sample of BSM before the changes had become obvious (Figure 12). This showed a slight increase in absorption at 275 mμ and may possibly have indicated the beginning of a change in structure.

The possible alterations in structure are considered in the Discussion.

Identity of BSM with Synthesized Material

It was decided to synthesize a series of 3-acyl esters of 7-hydroxycholesterol and to compare their properties with those of the naturally occurring BSM. Since the orientation of the postulated 7-hydroxyl group could not be determined it was decided to make the 7α-epimer as cholesterol is predominantly oxidized in this direction in biochemical attack.

The method of synthesis is described in Section 2B. A homologous series of esters was produced using the saturated fatty acids from C_{12} to C_{18} inclusive. Unsaturated acids could not be used because the method involved a bromination step. However, the oleyl and linoleyl derivatives were later synthesized

TABLE 4

R_F's OF 7α-HYDROXYCHOLESTEROL DERIVATIVES IN PAPER SYSTEM

Whatman no. 3, paraffin/$CHCl_3$:MeOH:paraffin

Compound	Chemically Synthesized	Enzymically Synthesized
7α-Hydroxy-cholesterol	0.88	-
Esters		
Laurate	0.56	0.55
Myristate	0.50	0.51
Palmitate	0.44	0.43
Stearate	0.36	0.35
Oleate	-	0.45
Linoleate	-	0.50

R_F BSM, 0.46; 0.51

enzymically (see Section 2C). Chromatographic characteristics of these esters were obtained and compared with those of BSM.

The mobility of BSM corresponded with that of the linoleyl and oleyl esters in all the three systems used (see Table 4 for R_F's on paper).

Note: BSM showed an elongated 'waisted' spot which probably consisted of two closely related esters. The centres of each of these corresponded with the linoleyl and oleyl esters. The R_F's of these esters are identical with those for the myristyl and palmityl esters respectively (see Section 2C), but by analogy with cholesterol esters, the first possibility is much more likely.

The colour reactions and staining characteristics of the chemically synthesized esters were identical with those of BSM.

The chromatographic evidence points very strongly towards the identity of BSM with the synthesized esters, particularly since the methods used depended upon different principles, two of them being partition methods and one absorption. Table 5 summarizes the evidence of identification.

Stability of BSM

Assuming the structure of BSM to be cholest-5-ene-3β-ol-7α-linoleate (or oleate) the most likely explanation of the disappearance of the material is that the dehydration occurred to give a diene. Studies of the chemically synthesized esters showed that reactions of this type occurred readily, giving intensively u.v.-absorbing Lifschůtz negative compounds. The synthetic compounds were unstable in the presence of hot ether or on exposure to u.v. radiation. The effect of the latter was to produce, transitorily, material which was u.v.-absorbing (λ max. 232 mμ), but which was rapidly converted to non-u.v.-absorbing, yellow material. Neither of these products was Lifschůtz positive or PTA positive (details in Section 2B).

Possible Reasons for the Sporadic Occurrence of BSM

The instability of the synthetic compounds under the conditions noted above would indicate that BSM was found only sporadically because it can be easily destroyed under conditions which might exist during extraction procedures. Ether is widely used in lipid extractions and its presence might account in part for the fact that

TABLE 6.

ATTEMPTED ARTIFACTUAL OXIDATION OF CHOLESTEROL ESTERS

In no case was BSM produced

Substrate	Conditions
Cholesteryl esters from human serum	Ageing - samples taken from ethanolic-acetone solution at intervals up to 6 months
Cholesteryl esters from human serum	Incubated at 37° in presence of Cu^{++}, H_2O_2, O_2 up to 60 hr.
Cholesteryl esters from human serum	Incubated with lipoxidase (see Section 2C)
Human plasma	Air bubbled through petrol ether-ether solution for 1 hr.; also in presence of H_2O_2
Cholesteryl linoleate	Incubated with various factors:- haemolysed plasma; Cu^{++}; Co^{++}; H_2O_2
Cholesteryl linoleate	Incubated with lipoxidase
Cholesteryl linoleate	Air bubbled through for 24 hr.
Cholesteryl stearate	Air bubbled through for 24 hr.

the compound has never been described previously.

It was not thought likely that the compound was an oxidation artefact since care was taken to try to eliminate oxygen from the procedures used. Check experiments were performed in which plasma which was BSM negative was deliberately exposed to oxidative conditions in order to try to produce the material artefactually, but this was never achieved. The list of conditions tried is seen in Table 6.

The effect of diet on the occurrence of BSM in the serum was not shown clearly (Table 2). In the rat the presence of the material did not appear to bear any relation to the fat content of the diet, nor to the presence of compounds which are known to affect cholesterol metabolism such as thiouracil.

In man, on the other hand, there are several instances of BSM occurring in sera of patients following administration of an unsaturated fat diet, whereas the compound had not been detected previously, nor was it present after the diet had been discontinued for one month. Unfortunately, practical considerations prevented further investigation of this phenomenon; the diet concerned was unpalatable and tedious to prepare.

Additional Note on the Chromatographic Behaviour of Acyl Esters of Vitamin A

During the course of routine paper chromatography of rat liver extracts a spot was sometimes observed which did not correspond in mobility to BSM, but appeared to stain blue. However, the stain faded rapidly to a dull grey-lilac colour.

The material giving this stain was eluted from silicic acid in a way similar to BSM, but was eluted considerably later from the reversed phase column. It could be detected by means of a Lifschütz reaction which did not follow the normal time-course of colour development. The reaction given was very intense and fleeting; the colour had disappeared completely after two to three minutes.

When the cuts containing the new material were evaporated, a sweet, sickly smell was given off which was similar to the classical description of the smell of vitamin A.

When a sample of vitamin A was investigated, it failed to give either of the colour reactions and had a much higher R_F on paper. A sample of vitamin A palmitate was obtained and this corresponded in every respect with the material from the liver extracts. The

TABLE 7

R_F's OF VITAMIN A DERIVATIVES

IN PAPER SYSTEM

Compound	R_F	Method of Detection
Vitamin A	0.85	Faint yellow colour u.v. absorbant
Vitamin A palmitate	0.29	Faint yellow; u.v. abs.; fleeting grey-blue PTA stain
7α-Hydroxy-cholesterol	0.88	Blue PTA stain
7α-Hydroxy-cholesterol palmitate	0.45	Blue PTA stain

latter was shown to produce vitamin A on hydrolysis, and also gave the correct u.v. absorption maximum at 325 mμ. (see Table 7).

This observation was of interest because of the superficial chromatographic similarity with BSM and also because no report has been published of the positive Lifschütz reaction with a vitamin A derivative. This is presumably because the unesterified vitamin fails to give the reaction.

Another appurtenance to the cholesterol cuts from rat liver was material which was subsequently shown to be ubiquinone. This material was strongly absorbing in u.v. light with selective maximum at 275 mμ. Ubiquinone did not give a positive Lifschütz reaction or PTA stain.

CONCLUSIONS

The routine examination of lipid extracts by chromatographic means revealed a new material which was Lifschütz positive. The structure of the material was shown to be a 3-acyl ester of 7α-hydroxycholesterol. If the material is a naturally occurring metabolite of cholesterol (and attempts to produce it artefactually were not successful), it is of considerable interest

as such compounds have not been described previously. The ester could have arisen by esterification of 7α-hydroxycholesterol, or by oxidation at the 7-position of a cholesterol ester. The metabolism of cholesterol esters by any means other than a hydrolytic enzyme has not been reported, but it may be that the presence of the ester link confers special properties upon the cholesterol molecule.

These possibilities are considered in the Discussion, together with other possible roles for the material, particularly with respect to its remarkable behaviour in response to ultra-violet irradiation.

SUMMARY

Material was isolated from serum, skin and liver in the rat, and from serum and leucocytes in man, which was shown to be identical to 3β-acyloxy-cholest-5-en-3β:7α-diol, when the acyl residue was linoleyl or oleyl. Identification was on the basis of colour reactions and of three types of chromatography, with additional information from both infrared and ultraviolet absorption spectra. Reasons are discussed for

the sporadic occurrence of the material, which was found in fourteen investigations out of a total of twenty-three.

Attempts were made to show that the material was not produced by autoxidation during the extraction process, and that it might therefore be of biochemical significance as a possible oxidation product of esterified cholesterol.

Other compounds which were associated with the material during chromatography included esterified vitamin A and ubiquinone.

SECTION 2B

THE CHEMICAL ESTERIFICATION OF 7α-HYDROXYCHOLESTEROL (Cholest-5-en-3β:7α-diol) AT THE 3β-POSITION BY VARIOUS FATTY ACIDS

EXPERIMENTAL

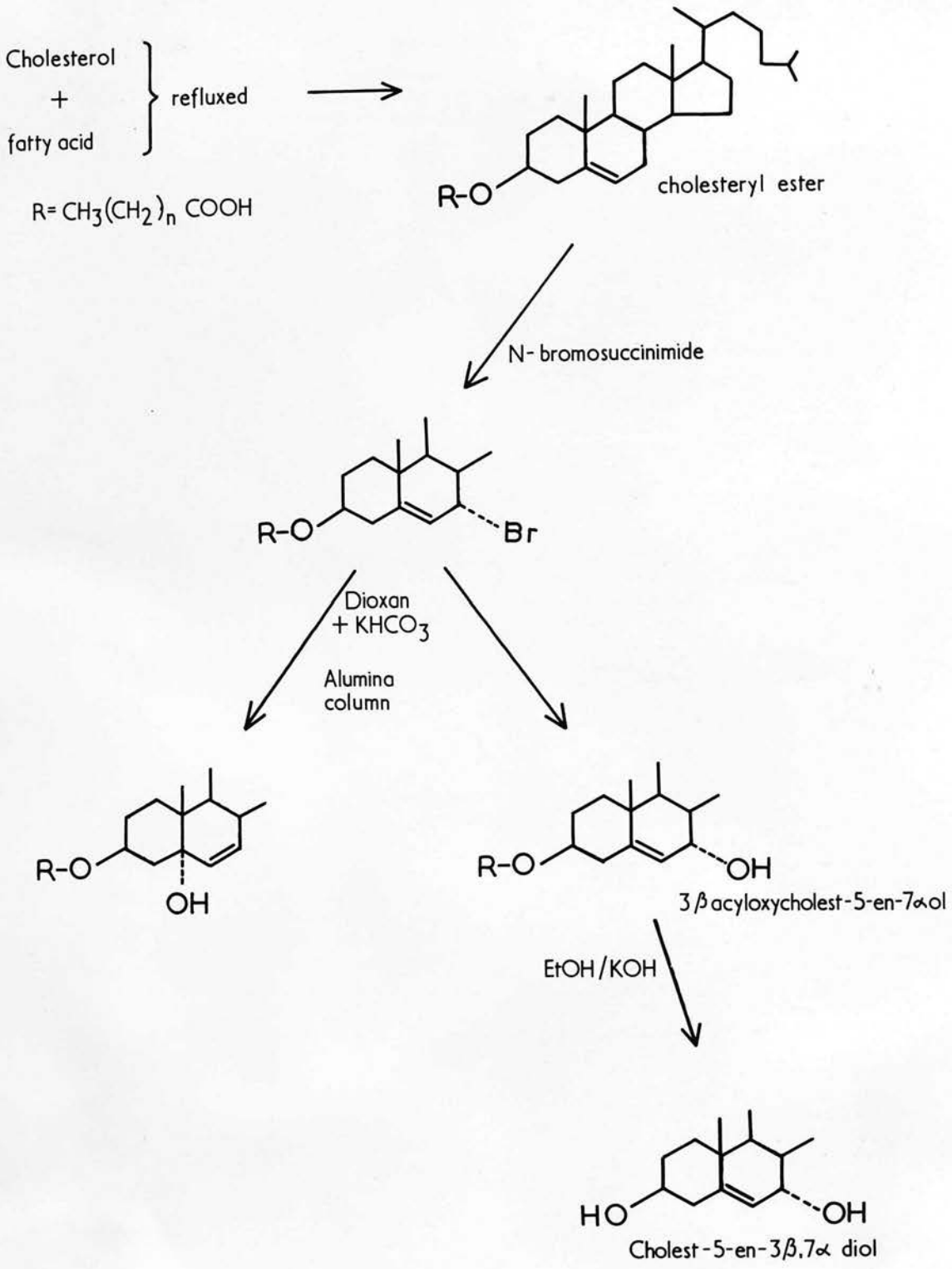
The syntheses to be described were undertaken in order to produce compounds which might be identical with the material isolated from natural sources (see Section 2A).

The synthesis of these esters is described in detail in this Section since no account has been published, except in the case of the acetyl ester. Because there were no recorded data, purification and characterization of the compounds were difficult, but eventually the series of esters from lauryl to stearic were synthesized, albeit in low yield.

The method chosen was a modification of that described by Henbest and Jones (1948) for the acetyl ester and depended upon a selective attack by N-bromosuccinimide at the 7α-position of cholesterol which had previously been esterified at the 3β-position. The 7-bromo compound was

FIG. 13

Synthesis of 3β acyl esters of 7α hydroxycholesterol



treated with potassium bicarbonate, which resulted in replacement of the bromine atom by a hydroxyl group. The product was isolated on an alumina column, and was purified by repeated recrystallization (see Figure 13).

Preparation of Cholesterol Esters (laurate, myristate, palmitate stearate)

These were synthesized from cholesterol (Weddell) and the appropriate fatty acid (Unilever) by the method of Page and Rudy (1930).

Cholesterol (30 g. 0.075 mole) was heated with 0.15 mole of the appropriate fatty acid for 2 hours under reflux at 200-210°C. The material was dissolved in ether and washed with 10% aqueous sodium bicarbonate. It was then recrystallized from ether by the dropwise addition of ethanol.

Melting points were as follows:-

	Observed	Reported	
Cholesterol laurate	88-90	92	Bladon, 1958
Cholesterol myristate	68-69	73	
Cholesterol palmitate	75-76	75	
Cholesterol stearate	74-75	71	

Note: The reported melting points vary since the esters soften several degrees before they melt. The temperature of liquefaction is usually taken as the melting point, but clarification of the liquid may not take place until a considerably higher temperature.

Each ester was eluted as a single peak from a silicic acid column with 2% chloroform in petroleum ether and ran as a single spot in the paper chromatographic system.

Bromination of Esters

The bromination step was performed using a 250 ml. 3-necked, round bottomed flask. The ester was dissolved in petroleum ether (60-80°C) and allowed to reflux, whilst being stirred mechanically; meanwhile the N-bromosuccinimide was quickly added through the third neck of the flask, and was washed through with a little of the petroleum ether which had been retained for this purpose. Refluxing continued for one hour during which time the solution became intensely yellow and a white crust of succinimide was deposited on the wall of the flask. This deposit was removed by filtration at the end of the reaction and the petroleum ether filtrate was

evaporated *in vacuo*. The reddish-brown gum was dissolved in the minimum of acetone and left at 0°C overnight, when white crystals were formed. These were filtered off and washed with acetone chilled to -20°C, and recrystallized by dissolving in acetone and chilling to approximately -40°C with solid carbon dioxide.

The bromo compounds obtained as a result were white and crystalline but very rapidly turned brown on exposure to air. In view of the extremely rapid decomposition of the compounds, elemental analysis and melting points were not performed as it was unlikely that these would be reliable.

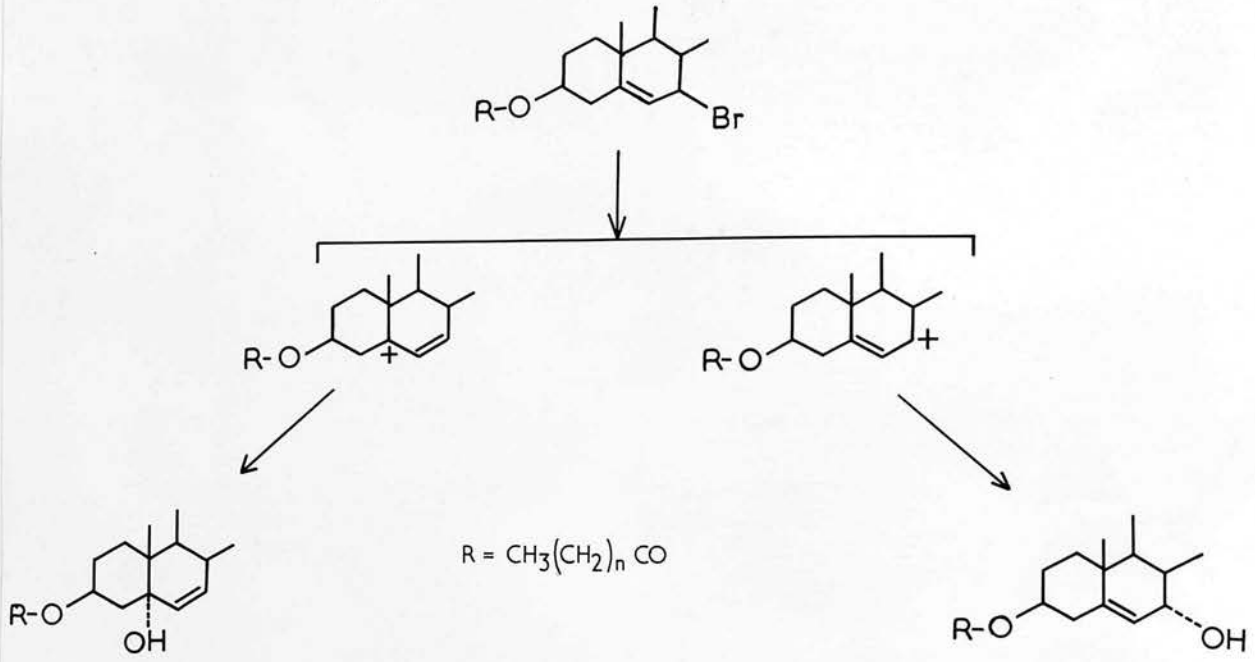
Details of Bromo Esters

Cholesterol ester	Weight g.	N-Bromo-succin-imide g.	Petroleum ether ml.	Yield of bromo-com-pound g.	Notes
Stearate	30	10.5	104	25	beige
Palmitate	30	10.2	100	30	white
Myristate	30	9.9	97	27	[x] reddish-brown
Laurate	25	8.0	80	21	white

[x] Difficult to crystallize

FIG. 14 A

Mechanism for the Formation of Isomeric 5 and 7 hydroxyl derivatives

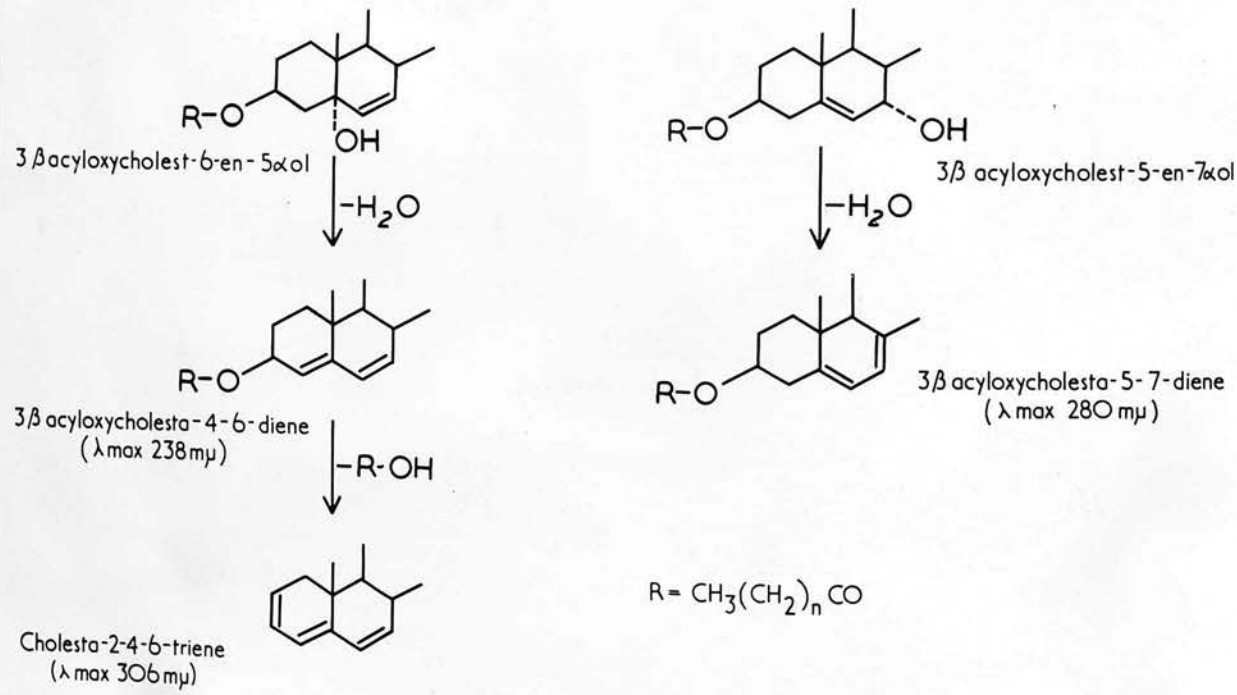


3β acyloxycholest-6-en-5α ol

3β acyloxycholest-5-en-7α ol.

FIG. 14 B

Dehydration Products from Isomeric Hydroxycholesterol esters



Partial Hydrolysis of the Bromo-Esters

The bromine atom at the 7α-position is extremely reactive, making selective partial hydrolysis possible. However, two products invariably result. Henbest and Jones (1948) suggest that loss of the bromine atom results in the formation of a mesomeric carbonium ion which may then be converted to either the 5- or the 7-hydroxy compound (see Figure 14). The two compounds were easily resolved in the case of the acetyl ester, but for the long chain esters, differences in polarity between the two forms were less marked. Further difficulties also arose by the formation of unsaturated compounds, probably dehydration products, of both forms.

The bromo-esters were dissolved in dioxan and allowed to react for 20 hours at room temperature with a solution in water of potassium bicarbonate.

A precipitate of potassium bromide formed and was filtered off; the filtrate was extracted with ether and concentrated. The concentrates were then submitted to column chromatography on alumina.

Cholesterol ester	Bromo-compound g.	Dioxan ml.	Potassium bicarbonate g.	Water ml.	Product g.
Stearate	24	210	10.5	42	12
Palmitate	29	250	12.0	50	20
Myristate	26	220	10.5	44	23
Laurate	20	160	8.0	33	17

The chromatographic procedure will be described for each ester individually, as the details differed slightly.

Column Chromatography

7α-Hydroxycholesterol Laurate

Column 1 - 185 g. of alumina (Peter Spence grade O) weakened with 4.5% water (8.3 ml.)

Two batches obtained:-

a) eluted by 1100 ml. of benzene (not further investigated)

b) eluted by 400 ml. 10% ether in benzene. Yield 9 g.

Column 2 - 'b' rechromatogramed on 450 g. of alumina, weakened as above, gave products as in the Table:-

Designation	L_5	L_6	L_7
Elution characteristics	200 ml. benzene	700 ml. 10% ether in benzene	300 ml. 25% ether in benzene
Colour with PTA	pink	blue	blue
R_F (paper system)	0.03 0.17	0.43 0.58	0.58
m.p. °C	82-88	98-100	101-103

L_7 taken for carbon and hydrogen analysis and infrared analysis, yield 1 g., low, due to repeated recrystallization from methanol.

7α-Hydroxycholesterol Myristate

Material divided into two batches.

Batch 1 (12 g.) - Run on 600 g. of alumina (5% water).

Resolved into - M_1 eluted with 600 ml. of benzene; low R_F on paper and very complex - retained for investigation (9 g.)

Batch 1 (contd.) M_2 eluted with 10% ether in benzene; contained some higher R_F material (1 g.). Recrystallized from methanol (500 mg., m.p. 52-56°C).

M_2 rechromatographed on 50 g. of alumina (6% water) resolved into:-

M_3 eluted with 500 ml. of benzene; low R_F (400 mg.), discarded.

M_4 eluted with further 500 ml. of benzene (100 mg.) recrystallized from methanol (50 mg., m.p. 96-99°C).

Retained.

Batch 2 (11 g.) Run on 550 g. of alumina (5% water).
Resolved exactly as above.
Produce M_5 (70 mg., m.p. 96-100°C. M_4 and M_5 were pooled and designated M_6. Yield after recrystallization from methanol 80 mg. (m.p. 97-99°C). (M_6 retained for analysis.)

7α-Hydroxycholesterol palmitate

12 g. run on 600 g. of alumina (4% water). Resolved into:-

P_1 eluted with 750 ml. of benzene (retained for investigation); low R_F material, very complex, Lifschütz positive, PTA negative;

P_2 eluted with 1,000 ml. 10% ether in benzene; one spot; R_F 0.36;

P_3 eluted with a further 500 ml. of 10% ether in benzene; contained two spots; R_F 0.17 and 0.36;

P_4 eluted with a further 1, 250 ml. of 10% ether in benzene; very complex; discarded.

P_3 retained and recrystallized from methanol. Yield about 50 mg., m.p. 55-56°C.

7α-Hydroxycholesterol Stearate

12.5 g. run on 600 g. of alumina (2½% water). Resolved into :-

S_1 7 g. of material of low R_F; very complex; showed PTA blue and pink spots on paper - discarded;

S_2 2 g. of brown, single, intense spot; higher R_F - retained.

S_2 rechromatographed on 120 g. of alumina (5% water). Resolved into:-

S_3 eluted with 1,000 ml. of benzene; complex - 1.8 g.

S_4 eluted with 1,250 ml. 5% ether in benzene - 200 mg.; recrystallized from methanol - m.p. 72-74°C; run on paper as slightly 'waisted' spot - 100 mg. of S_4 retained for analysis.

RESULTS

A summary of the properties of the synthesized esters is given in Table 8.

The elemental analyses fall within the limits normally considered acceptable.

TABLE 8

CHARACTERISTICS OF 3β-ACYL ESTERS OF 7α-HYDROXYCHOLESTEROL

Data for 7α-hydroxycholesterol and the acetyl ester are included for comparison.

Compound	Elemental Analysis Calculated	Found	Melting Point °C	Infrared Abs. Maxima	R_F in Paper System
[x] 7α-Hydroxycholesterol	80.52	80.22	178	3600) equatorial 1052) hydroxyl	0.88
	11.52	11.16		3600) axial 1012) hydroxyl	
[x] Acetyl ester	78.33	78.38	135	1240) acetyl 1734) ester	0.70
	10.88	10.75		3620) axial 1012) hydroxyl	
Lauryl ester	C 80.08	79.82	101-103	1174) 1734) ester	0.58
	H 11.72	11.33		3620) axial 1015) hydroxyl	
Myristyl ester	C 80.33	79.89	97-99	1174) 1734) ester	0.51
	H 11.84	11.69		3620) axial 1015) hydroxyl	
Palmityl/					

Contd.

Melting points show a gradation in the direction which would be expected when ascending a homologous series, with the exception of the palmityl ester. This anomalous melting point may possibly be accounted for by the weak ethereal impurity detected on infrared analysis, but all attempts to raise the melting point by repeated recrystallization were unsuccessful. Infrared absorption data are satisfactory, but for the exception of the palmityl ester, and the behaviour on paper chromatograms shows the pattern which might have been predicted - the increasing chain length of the fatty acid renders the molecule less polar, and hence the R_F is lower in the reversed phase system used.

With no published data for comparison, it is not easy to establish criteria of purity for the synthesized esters. As far as can be determined however, the compounds appear to be reasonably pure with the possible exception of the palmitate.

Investigation of Low Yields

It was obvious when reviewing the syntheses from a quantitative point of view that the desired compounds were undergoing some change in structure to give a form which was apparently

more stable, and of lower polarity. This process appeared to be accelerated by the alumina column treatment, and the ratio of unwanted to wanted material seemed to increase with the chain length of the acid. It was thought likely that the alkalinity of the alumina was affecting the process, and so columns were run using acid-washed alumina, and indeed the yield was higher, although the unwanted material still appeared in lower concentration.

The 'early cuts' from the myristyl and palmityl preparations had been retained, and these were investigated. The results are described in the following paragraphs.

Investigation of M_1 (early cuts eluted by benzene)

The material appeared to fall into two peaks (M_{1A} and M_{1B}) as shown by the semi-quantitative Lifschütz tests which were performed on the cuts. Both peaks were yellow and strongly u.v.-absorbing.

M_{1A} λ max. 230 mμ (probably 4-6 diene)

M_{1B} λ max. 280 mμ with inflexion at 240 mμ (probably 5-7 diene)

M_{1B} (recrystallized from methanol)
λ max. 240 mμ, m.p. 46-48°C

Note: M_{1B} probably reverted to a 4-6 diene on recrystallization.

Investigation of P_1 (early cuts eluted by benzene)

Absorption in Ultraviolet Light

Intense absorption, λ max. 305 mμ (probably 2-4-6 triene). Exposure to solutions of different pH (HCl and NaOH) did not affect the absorption.

Crystallization

The material which was a yellow gum was partially soluble in methanol. The solution was chilled and a white precipitate was obtained (P_5).

The yellow residue was recrystallized four times from ethyl acetate to give white crystals (P_6) and a yellow supernatant (P_7). P_5 showed an absorption spectrum unchanged from the starting material. P_6 showed no selective absorption at 305 mμ, but two new peaks, a major one at 240 mμ and a minor one at 270-280 mμ. P_7 showed selective absorption at 240 mμ and slight inflexion at 280 mμ.

From the u.v. absorption data on the early cuts from the myristyl and palmityl columns it seems clear that dehydration reactions had taken place on the alumina. Absorption in the range 230-240 mμ is indicative of derivatives of cholesta-4-6-diene, around 280 mμ cholesta-5-7-

diene and at 305 mμ cholesta-2-4-6-triene (Dorfman, 1953). In the last case, the fatty acid residue presumably had been eliminated.

The reactions are summarized in Figure 14b.

Investigation of Solution of 7α-Hydroxy-Cholesterol Stearate

A standard solution of the material in chloroform had been made up for chromatography and stored in a flask in a dark cupboard. The solution had turned yellow and gave much less Lifschütz chromogen than was appropriate to its concentration, and this continued to diminish on storage. Paper chromatography showed that as the original material diminished, a new spot appeared which was u.v.-absorbing and of lower R_F.

An absorption spectrum showed that the maximum was at 232-234 mμ with a slight inflexion at 280 mμ.

An attempt was made to produce the above change by exposing a solution of the ester to ultraviolet light, sampling at intervals, and investigating the samples by u.v. absorption and paper chromatography. The results were as follows:-

Effect of Ultraviolet Irradiation on 7α-Hydroxy-Cholesterol Stearate

Time min.	Paper Chromatogram	u.v. Absorption λ max. mμ
0	blue spot	slight trace 234, 239
20	blue spot vanished u.v. absorption spot, low R_F	selective absorption at 236
40	u.v. absorption spot vanished u.v. yellow fluorescent spot, high R_F	very slight abs. at 236 increase in general absorption
60) 120)	very intense yellow fluorescent spot almost at solvent front	no selective abs. increase in general absorption

Material was produced as a result of u.v. irradiation which had a u.v. absorption spectrum compatible with a cholesta-4-6-diene structure. This rapidly gave way to a yellow compound with no selective absorption. Apparently the continued absorption of u.v. light by the molecule had resulted in a drastic change in its structure. If the 4-6-diene structure had been

in equilibrium with a 5-7-diene structure it is possible that a ring opening reaction may have occurred similar to that involved in the activation of vitamin D.

SUMMARY

A homologous series of 3β-acyl esters (lauryl to stearyl) of 7α-hydroxycholesterol has been prepared. The synthesis and characteristics of the compounds are described. Special features noted during the synthesis include:-

a) Low yields due to two factors; firstly, the possibility of producing the 3β-5α-diol, and secondly, the formation of dehydration products of both the 3-5-diol and the 3-7-diol.

b) The formation of these products was accelerated by the use of alumina columns, and was more marked with an increase in chain length of the fatty acid.

c) The sensitivity of the compounds to ultraviolet light - after 20 minutes' exposure the stearyl ester was completely destroyed.

SECTION 2C

ENZYMIC EXPERIMENTS INVOLVING THE 3β-ACYL ESTERS OF 7α-HYDROXYCHOLESTEROL

The work to be described in this Section falls into three categories according to the enzyme system used.

a) Lipoxidase.

b) Hepatic and pancreatic cholesterol esterase.

c) Hepatic cholesterol oxidizing systems.

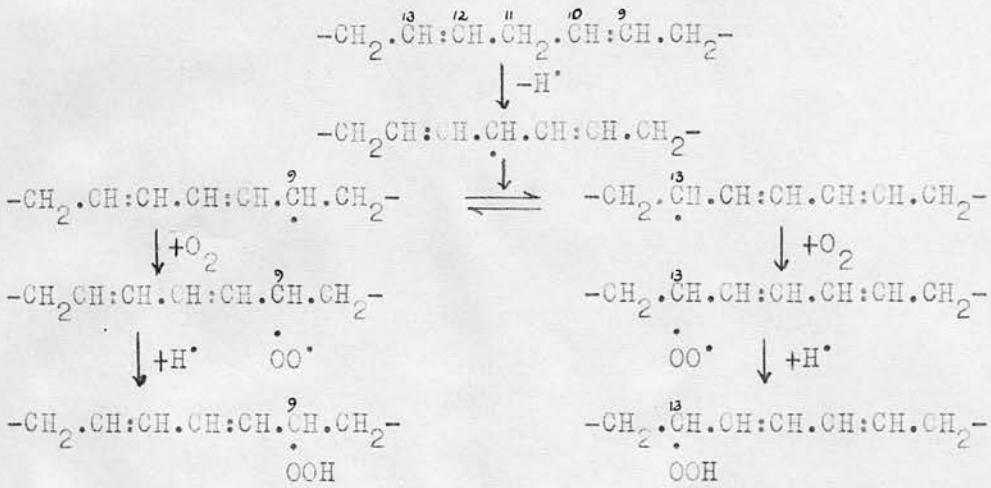
a) Lipoxidase

Lipoxidase is a plant enzyme which is found in very high concentration in soya beans (Craig, 1936; Sumner, 1943; Andre and Hou, 1932). The enzyme attacks long chain fatty acids with an all cis methylene interrupted series of double bonds, causing hydroperoxide formation, and a shift of the double bonds to the conjugated position.

Holman and Burr (1945) showed that after lipoxidase attack the oxidized acid absorbed u.v. light maximally at 235 mμ. A mechanism for the attack was suggested by Bergstrom and Holman

FIG. 15

Mechanism of Action of Lipoxidase(as proposed by Bergstrom and Holman.)



(1948) (see Figure 15). The enzyme was obtained pure from soya flour by Theorell, Holman and Akeson in 1947.

EXPERIMENTAL

The purpose of the following experiments was to establish whether or not lipoxidase could attack the fatty acid moiety of cholesteryl linoleate. It was thought that the hydroperoxide which would be formed at the C_{13} position of the acid might stimulate oxidation of the steroid nucleus at C_7 (see Figure 19). If this dual oxidation did occur, it might be one reason for the increased turnover of cholesterol in the presence of linoleic acid (see Section 1). This possibility is described in more detail in the Discussion.

There exist well established methods for the lipoxidase reaction, and that of MacGee, Matson and Beek (1958) was adopted with slight modification. Attempts to produce the enzyme from soya bean meal were not successful, probably the meal had been heat treated, and so a commercial preparation was used (Light's). The assay method consists of measurements of the absorption of

ultraviolet light at 234 mμ, this being the wavelength at which the conjugated double bond system absorbs.

The reaction mixture consisted of 2 ml. of substrate solution containing approximately 0.3 μmole linoleate in borate buffer, pH 9.0 (0.2M), and 0.3 ml. of enzyme solution (150 μg./ml. in borate buffer, pH 9.0). (Because of the low solubility of linoleic acid, the potassium salt is normally used.) This reaction mixture was allowed to stand at room temperature for 30 minutes and then the extinction at 234 mμ was measured, using a similar mixture with boiled enzyme as blank. The reaction was stopped by the addition of 4 ml. of ethanol.

When cholesteryl linoleate was used the reaction mixture was retained after u.v. absorption measurement, extracted with chloroform, and this extract run on paper to scan for blue-staining spots.

The main difficulty encountered was the problem of preparing cholesteryl linoleate in a suitable emulsion for enzymic attack.

RESULTS

Despite the use of effective emulsifying agents, no attack by lipoxidase on cholesteryl linoleate could be detected.

When methyl linoleate was substituted activity was shown, indicating that the presence of an ester link was not exerting an inhibitory effect, and that the presence of emulsifying agents was not a complicating factor. The activity of the enzyme was always checked against potassium linoleate.

The following Table summarizes the conditions used. In no case was there any activity demonstrable, either by u.v. absorption or by paper chromatography.

Substrate	Additions to Reaction Mixture	
Cholesteryl linoleate	sodium taurocholate	(e)
	gum arabic	(e)
	glycerol monostearate	(e)
	Tween 40	(e)
	Tween 40 (e) + Cu^{++}	(o)
	Tween 40 (e) + Co^{++}	(o)
	Tween 40 (e) + H_2O_2	(o)
	Tween 40 (e) = haem	(o)
Human serum cholesterol ester fraction[x]	gum arabic	(e)

(e) emulsifying agents

(o) agents reported to catalyse similar oxidations

[x] After hydrolysis with potassium hydroxide, considerable activity was measured.

CONCLUSIONS

Lipoxidase did not attack cholesteryl linoleate. The explanation may be that the enzyme is specific for salts of unsaturated fatty acids, and esters of relatively small bulk. The steroid nucleus may produce steric hindrance to the enzyme.

A related reason may be that the physical state of the substrate did not allow approach of the enzyme. It is of interest that the isolated plasma cholesterol fraction was only attacked after hydrolysis.

b) Cholesterol Esterase

Cholesterol occurs in most tissues of the body in both the free and esterified forms, which attain equilibrium rapidly. Enzymes catalysing the formation and hydrolysis of cholesterol esters with aliphatic acids have been described in several tissues. The need for esterification of cholesterol has never been elucidated, but it may be significant that in those tissues (e.g. brain) where cholesterol is inert, it is almost entirely unesterified, whereas in organs where the turnover of cholesterol is rapid, esterification occurs to a relatively large extent.

Cholesterol esterase activity has been reported in liver, pancreas, intestinal mucosa and plasma. A review of this work is contained in a chapter by Gould and Cook (1958).

The enzyme obtained from pancreas is by far the most active, and most work has been done using preparations of the organ. Commercial pancreatin is the most common source of the enzyme, which was purified by Hernandez and Chaikoff in 1957. Their final product showed two peaks on electrophoresis, but further resolution was not possible. The ratio of hydrolytic to synthetic activity remained constant throughout the purification process, which has led the authors to postulate that one enzyme is responsible for both activities.

These authors reported that the optimum pH for the hydrolytic reaction was 6.8, and for esterification 6.2. Similar results were obtained by Swell and Treadwell (1950) and Korzenovsky, Rust and Diller (1955). Work has been reported by these three sets of workers on the effect of different fatty acids and different sterols on the enzyme activity. This work will be summarized later in the Section (p. 95).

Liver cholesterol esterase activity was first described by Sperry and Brand (1941), but subsequent reports have produced conflicting data. Byron, Wood and Treadwell (1953) failed to show esterification using similar conditions to the pancreatic enzyme, but Fredrickson (1956b) showed that up to 25% of cholesterol-26-^{14}C became esterified on incubation with mouse liver mitochondria.

Swell, Boiter, Field and Treadwell (1955) were unable to show cholesterol esterase activity in liver, but Mukherjee, Kunitake and Alfin-Slater (1958) described a system with considerable esterification activity.

The following experiments were performed using either liver or pancreatin as a source of cholesterol esterase. In neither case was successful purification of the enzyme achieved, and the experiments can only be strictly classified as enzymic in that the factor causing activity was heat labile. The object was to ascertain whether esters of 7α-hydroxycholesterol were suitable substrates for cholesterol esterase.

The practical difficulties which had to be overcome were:-

i. Preparation of a suitable emulsion of the substrate.

ii. Development of micro-methods for the recovery and estimation of substrate and product.

The first problem was solved by the use of either Tween 40 or plasma albumin. The latter was considered preferable since it was more physiological, but another difficulty occurred because the albumin obtained was contaminated with traces of fatty acids which interfered with the reaction. These were removed by extraction with iso-octane at 4°C (Goodman, 1957). As far as could be detected, there was no interference in the reaction when Tween was used, but as the extracted albumin proved satisfactory, this was the method adopted.

The problem of assay was a tedious one. The procedure which proved most satisfactory, although very laborious, consisted of extracting the reaction mixture with ethanol/acetone (1:1) and then twice with ethyl acetate. The combined extracts were concentrated and run on small silicic acid columns, the appropriate cuts being evaporated to dryness for estimation by a colorimetric method. Experiments showed that,

with care, a constant recovery of 90% could be obtained for both substrate and product. Details are given in the Appendix.

The earlier experiments were qualitative; the extracts were run on paper for identification of products, but these were followed up by quantitative studies as far as time permitted.

The following conditions were investigated:-

Liver

i. Hydrolysis of cholesterol acetate and cholesterol stearate.

ii. Hydrolysis of 7α-hydroxycholesterol acetate and stearate.

Pancreatin

i. Establishment that both hydrolysis and esterification do occur when the 7α-position of cholesterol is hydroxylated.

ii. Comparison of the hydrolysis of cholesterol acetate and 7α-hydroxy-cholesterol acetate (3β-acetoxy-cholest-5-en-7α-ol).

iii. Effect of fatty acid chain length on both hydrolysis and esterification reactions involving 7α-hydroxycholesterol.

Determination of pH optima was not undertaken as the preparations were not purified. Buffers at the pH values recommended by Hernandez and Chaikoff (1957) were used.

Liver

EXPERIMENTAL

Incubation mixture:-

3 g. of rat liver homogenized with 27 ml. of 0.25 M sucrose containing 3 mg. of nicotinamide per ml. - cell debris and nuclei spun down (Schneider, 1948).

Each tube contained 2 ml. of the homogenate. In these experiments the substrate was added as an emulsion with Tween 40 (approximately 1 mg. of sterol substrate in a solution containing 1 g. of Tween 40 in 100 ml. of buffer). Enzyme blanks contained boiled homogenate.

The reactions were stopped by the addition of 25 ml. of ethanol/acetone (1:1, v/v). The tubes were centrifuged and the residues re-extracted twice with 25 ml. portions of chloroform/methanol (3:1, v/v).

i. Incubation of Cholesteryl Acetate and Cholesteryl Stearate (quantitative)

In the case of the cholesterol esters, these extracts were concentrated; each was dissolved in 10 ml. of acetone/alcohol from which four 2 ml. portions were removed; of these, two were hydrolysed with potassium hydroxide, two remained unhydrolysed, and all were then measured for cholesterol content by the Sperry and Webb method

RESULTS

Measurements of Cholesterol in µg. (1/5 original tube)

Time (hours)	0	3	6
Cholesteryl acetate			
total cholesterol (i.e. free and unesterified)	366	300	322
'free' cholesterol	169	211	293
total-free (i.e. cholesterol combined as esters)	197	89	29
Cholesteryl stearate			
total	164	182	151
free	147	160	125
total-free	17	22	26
Liver - no substrate			
total	137		140
free	144		137
total-free	-7		3

These results indicate that cholesteryl acetate was hydrolysed almost completely in six hours, the loss in esterified cholesterol being almost entirely accounted for by the increase in unesterified cholesterol.

No hydrolysis occurred in the boiled preparation. The cholesteryl stearate experiment showed no positive results; it appeared that very little of the ester had been extracted, as little difference in cholesterol reading was observed between these tubes and those containing only liver and no substrate. The readings obtained for liver alone indicate that in this case there was virtually no endogenous cholesterol ester. This is unusual as rat liver normally contains up to 10-20% of the cholesterol esterified.

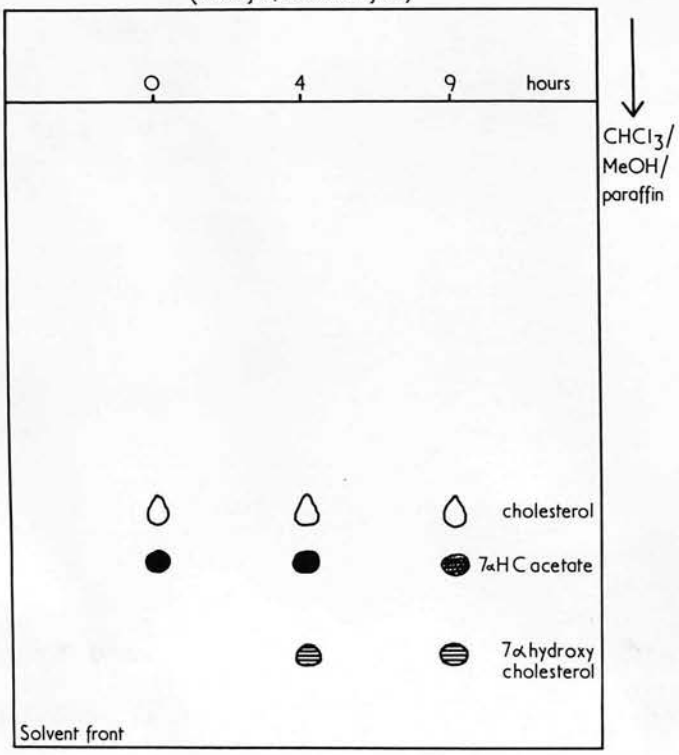
Cholesterol stearate does not appear to have been hydrolysed as, despite the failure to recover the substrate, there is no increase in unesterified cholesterol.

It is possible that the sucrose residue prevented complete extraction of the long chain ester.

In the preparation used there appeared to have been no net synthesis of cholesterol. A check experiment with cholesteryl acetate showed

FIG. 16 A

Hydrolysis of 7α hydroxycholesteryl acetate by liver preparation
(tracing of chromatogram)



that the liver preparation was effective in the absence of sucrose so this was omitted from the following experiments, as its presence made the extraction process more difficult.

ii. Incubation of 7α-Hydroxycholesteryl Acetate and 7α-Hydroxycholesteryl Stearate (qualitative)

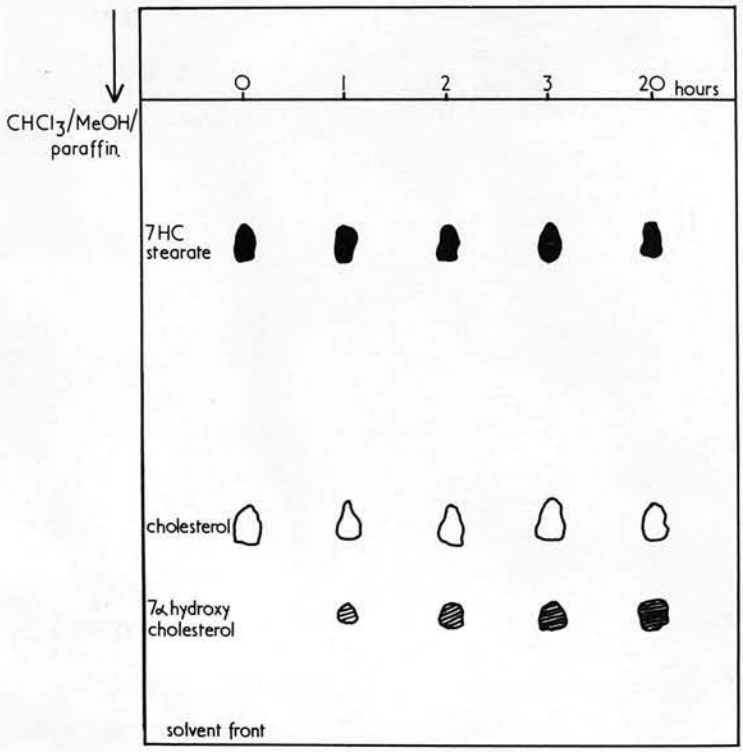
Homogenate conditions were identical with the previous experiments except for the omission of sucrose and differences in the times of incubation.

7α-Hydroxycholesteryl Stearate

The presence of the free alcohol was detectable after one hour's incubation with the homogenate, and the concentration increased with time until twenty hours, which was the length of the experiment. The spot for cholesterol was readily visible, but no 7α-hydroxycholesterol had been formed from cholesterol by autoxidation, nor had the ester been hydrolysed except in the presence of the homogenate. A diagram of the results is shown in Figure 16a.

FIG. 16 B

Hydrolysis of 7α hydroxycholesteryl stearate by liver preparation III



7α-Hydroxycholesteryl Acetate

Hydrolysis of the ester took place in the tubes containing unboiled homogenate. A trace of the free alcohol was visible after one hour, a considerable amount after four hours, and after nine hours the substrate had disappeared completely although there appeared to be no increase in the amount of 7α-hydroxycholesterol. Presumably the latter was being removed by further metabolic processes (see Figure 16A).

Pancreatin

EXPERIMENTAL

Incubation Conditions

Each tube contained:- 50 mg. of pancreatin in 2 ml. of 0.15 M phosphate buffer at pH 6.8 for hydrolysis and 6.2 for esterification and 1 mg.[x] sterol in 0.1 ml. ethanol except where stated to the contrary.

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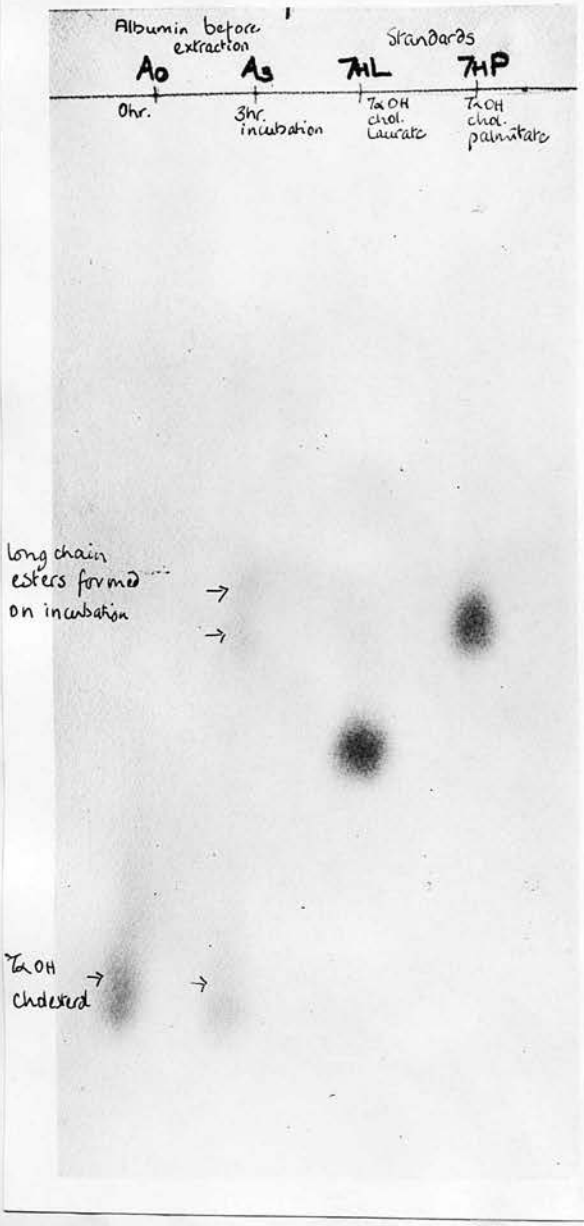
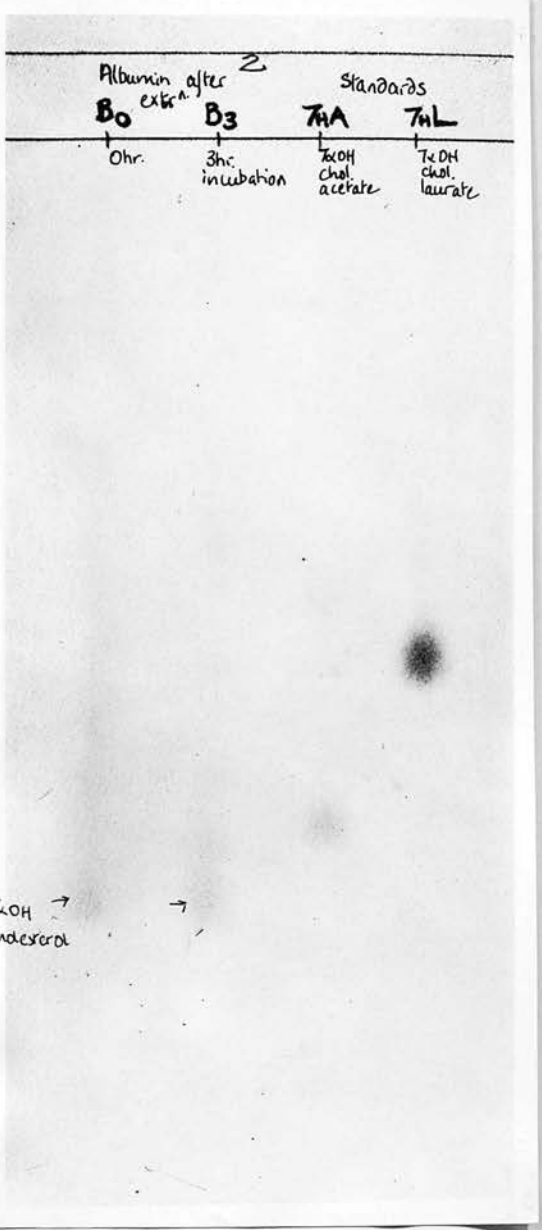
[x] 2.5 μmoles 7α-hydroxycholesterol. Weight of esters adjusted to be equivalent. All weights quoted refer only to sterol content.

FIG. 17


Albumin before extraction
Standards
A0
A3
7HL
7HP
0hr.
3hr. incubation
7α OH chol. Laurate
7α OH chol. palmitate
long chain esters formed on incubation
7α OH Cholesterol


Albumin after extn.
Standards
B0
B3
7HA
7HL
0hr.
3hr. incubation
7α OH chol. acetate
7α OH chol. laurate
7α OH Cholesterol

The ethanolic solution was slowly added to the pancreatin suspension using a micrometer syringe. The suspension was kept at 40°C and shaken during the addition until the ethanol had evaporated. The incubation was at 37°C for the times indicated in each experiment. The reaction was stopped by the addition of 20 ml. of acetone/alcohol (1:1).

In some experiments the sterol was added first to 1 ml. of a 1% solution in buffer of plasma albumin, and this was then added to the pancreatin which had been dissolved in only 1 ml. of buffer.

Since it proved difficult to remove all traces of fatty acid from the albumin, and the crude pancreatin suspension was sufficiently viscous to support the dispersed sterol, the use of the albumin was discontinued in the later experiments.

The complications arising from the use of albumin were evident in an early experiment when 7α-hydroxycholesteryl acetate was incubated. In addition to the spot for the free 7α-hydroxycholesterol, spots were also visible which corresponded to long chain esters of 7α-hydroxycholesterol (see Figure 17). The only

explanation for this was that the free alcohol had become re-esterified with fatty acids present either in the pancreatin itself or in the albumin. After lipid extraction of the albumin by the method of Goodman (1957), this phenomenon disappeared. Use was made however of this physiologically dispersed fatty acid to study the time course of the esterification reaction. The maximum concentration of ester was reached after two hours and appeared to stay constant up to six hours.

For the hydrolysis of 7α-hydroxycholesteryl acetate the maximum activity was observed after four hours.

It is difficult to provide satisfactory documentary evidence for the quantitative reaction as the chromatograms fade rapidly making photography a complicated procedure; tracings were therefore taken, and some of them are reproduced here to illustrate the results.

A. Esterification Reactions (pH 6.2)

Qualitative

1. Esters formed by incubation with human plasma albumin before and after extraction, and with Tween. See Figure 17.

2. Esters formed with stearic, oleic, linoleic, palmitic, myristic and lauric acids. Three hour incubation. R_F's of esters corresponded in each case with the standards. No standards were available for the oleate or linoleate. See Table 4.
The extracts were run again on paper after storage, when the linoleyl ester spot disappeared and a u.v. absorbing spot, non-Lifschutz positive appeared, running nearly at the solvent front. This material showed selective absorption at 275 mμ.

3. Esters formed with stearic, linoleic and acetic acids. Three hour incubation. Esterification appeared to be more ready with linoleic than with stearic acid. No reaction was observed with acetic acid.

Quantitative

1. Effect of Chain Length of the Fatty Acid on Esterification of 7α-Hydroxycholesterol

Time - 4 hours

1.7 mg. of substrate

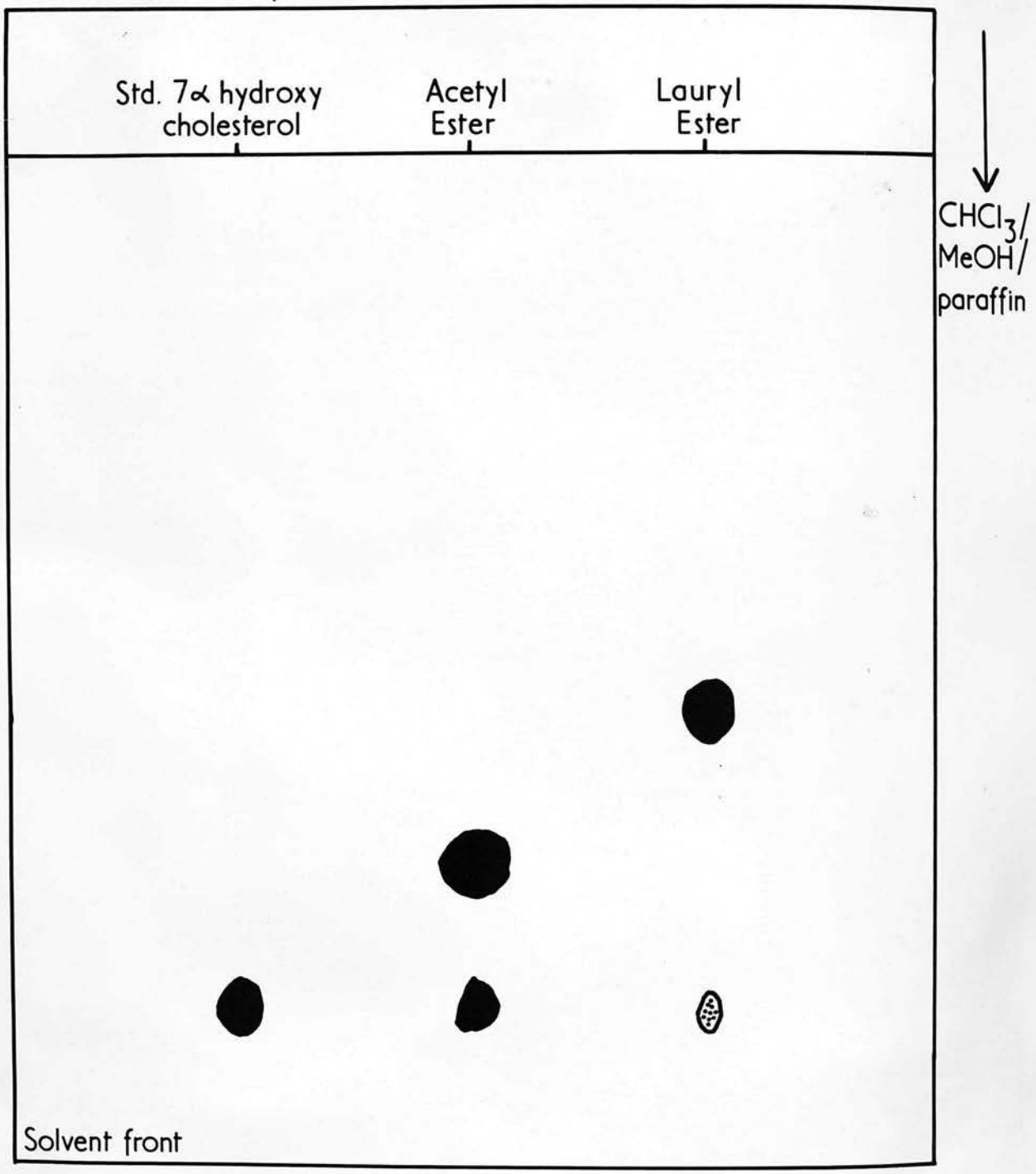
Acid	Hydroxy-cholesterol ester (μg.)	Hydroxy-cholesterol free (μg.)	% Esterification
Stearic	128	1,590	7.4
Oleic	210	1,500	12.3
Linoleic	240	1,400	14.6
Palmitic	125	1,520	7.6
Myristic	215	1,450	12.9
Lauric	205	1,480	12.0
Linoleic boiled enzyme	18	1,650	1.0

All from duplicate readings.

7α-Hydroxycholesterol was esterified most readily with linoleic acid; oleic, myristic and lauric acids were esterified to a slightly less extent, but stearic and palmitic acids only to half the extent of linoleic.

FIG. 18

Hydrolysis of Acetyl and Lauryl Esters of 7α hydroxycholesterol by Pancreatin (4 hour incubation)



B. Hydrolytic Reactions

Qualitative

1. Effect of Chain Length of the Acid on Hydrolysis

The acetyl and lauryl esters were used and were incubated for 5 hours. Both esters were hydrolysed, the acetate to a much greater extent. See Figure 18.

Quantitative

1. Comparison of the Hydrolysis of Cholesteryl Acetate and 7α-Hydroxycholesteryl Acetate

Time - 3.5 hours

1 mg. of sterol substrate

Time (hours)	Sterol Ester (μg.)	Free Sterol (μg.)	% Hydrolysis
Cholesteryl-acetate			
0	1,000	0	0
3.5	810	180	19
7α-Hydroxy-cholesteryl acetate			
0	960	60	5.8
3.5	425	550	44

7α-Hydroxycholesteryl acetate was hydrolysed much more readily than was cholesteryl acetate.

2. The Effect of the Chain Length of the Acid on Hydrolysis

i) Hydrolysis of 7α-Hydroxycholesterol Laurate and Myristate

Substrate (2mg. sterol)	Time (hr.)	µg. Sterol as Ester	µg. Free Sterol	% Hydrolysis
Acetate	0	2,000	18	1
	3.5	720	1,100	48
Laurate	0	1,940	0	0
	3.5	1,850	110	3.6
Myristate	0	2,050	0	0
	3.5	1,800	100	4.9

ii) Hydrolysis of the Lauryl and Stearyl Esters

Substrate (0.5 mg. sterol)	Time (hr.)	µg. as Ester	µg. Free Sterol	% Hydrolysis
Laurate	0	620	0	0
	22	440	95	15
Stearate	0	540	0	0
	22	340	110	20

There was apparent disappearance of the substrate to the extent of approximately 20%, presumably as a result of the long incubation.

The effect of chain length upon hydrolysis is not clear. The acetyl ester was hydrolysed

much more readily than any of the long chain ones, but in the range C_{12} to C_{18}, an increase in chain length appears to favour the reaction.

CONCLUSIONS

The cholesterol esterase present in both liver and pancreas can form and hydrolyse 3β-esters of 7α-hydroxycholesterol. No reports have been published describing these reactions although Hernandez and Chaikoff (1957) quote experiments by other workers claiming that a 7α-hydroxyl group prevented activity (see Discussion, p. 114). Korzenovsky, Rust and Diller (1955) stated that epicholesterol, coprostanol and 7-dehydrocholesterol were not suitable substrates for the enzyme. The experiments comparing hydrolysis of cholesteryl and 7α-hydroxycholesteryl acetates indicate that activity is much greater in the latter case.

The effect of chain length of fatty acid on the reactions is similar in some respects to that reported for cholesterol esterase.

Korzenovsky, Rust and Diller (1955) state that long chain unsaturated acids are more

readily esterified than are saturated ones, and that esters of short chain fatty acids are more readily hydrolysed. Hernandez and Chaikoff (1957) quote the following percentage figures for esterification, using oleic acid as standard:- oleic, 100; linoleic, 109; stearic, 44; palmitic, 28; myristic, 0. Apart from the last result, the order of activity shown is similar to that demonstrated for the hydroxylated esters.

It may be just by chance that the hydroxylated esters can follow an existing metabolic pathway for cholesteryl esters, but since the enzyme appears to be relatively specific, it is possible that there is some biochemical significance in the hydrolysis or synthesis of esters of 7α-hydroxycholesterol.

C. Cholesterol Oxidizing Systems in Liver

In vitro experiments on the oxidation of labelled cholesterol were first performed by Meier, Siperstein and Chaikoff in 1952. They were able to show the production of $^{14}CO_2$ from 26-^{14}C-cholesterol in rat liver slices. Similar results were obtained using mouse liver preparations by Anfinson and Horning (1953).

In 1956, Fredrickson investigated the oxidation products from 4-^{14}C-cholesterol in mouse liver, and found that about 15% of the label was present as acids not identical with cholic or deoxycholic acid; 7% was in 25- or 26-hydroxycholesterol, and 3% was in the form of cholesterol esters.

More detailed analysis of the products has been performed by Danielsson (1958) and Danielsson and Horning (1959), using reversed phase columns. By repeated chromatography, peaks from one column were resolved on another, and several new compounds were isolated (Danielsson, 1960 and 1961).

The experiments to be described were performed using rat or mouse liver mitochondria prepared according to Danielsson and Horning (1959).

The object was to see whether cholesterol esters were more readily oxidized than was cholesterol, and to determine whether cholesterol esters could be converted to 7α-hydroxycholesterol esters.

Unfortunately, due to delays in obtaining the labelled compounds which were to be used as substrates, these experiments are only of a

preliminary nature and adequate controls and recoveries were not performed in every case.

EXPERIMENTAL

Composition of Incubation Mixture

Each tube contained:-

Mitochondria, 3 ml. (from 5 g. of liver, suspended in sucrose/nicotinamide solution)

[x]Boiled liver juice, 3 ml.

Adenosine monophosphate (AMP) 0.014M, 1 ml.

Diphosphopyridine nucleotide (DPN) 0.01M, 0.5 ml.

Tris 0.05M, pH 7.8, 2 ml.

Substrate in 1% albumin solution, 1 ml.

The mixture was incubated at 37°C for 4 hours with constant shaking, and extracted according to the method of Danielsson and Horning. Fractionation was carried out using the Danielsson Type 3 Column.

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[x] The preparation of this curious component is described in detail by Danielsson and Horning. Without it, activity is very low.

1. Conversion of Cholesterol to More Polar Products

Cholesterol labelled randomly with ^{14}C, obtained by biosynthesis and purified by chromatography to constant specific activity (100,000 counts per minute per mg.).

500 µg. of cholesterol (50,000 c.p.m.) incubated with rat liver mitochondria.

Result:

	Total counts/min. recovered	
	Test	Blank
[x]Acidic fraction	1,032	105
[x]Neutral fraction	1,119	398
Total	2,150	503

The net conversion was 4% of which 1% was due to autoxidation to a more polar product.

[x] Nomenclature according to Danielsson:-

Acidic fraction - mainly cholic acid and chenodeoxycholic acid

Neutral fraction - mainly cholestenediols and cholestane-diols and triols.

2. Oxidation of Cholesteryl Linoleate at the 7-Position

The cholesterol linoleate in which cholesterol was labelled as in the previous experiment, was synthesized using the method of Page and Rudy (1930). Specific activity was 20,000 c.p.m./mg. Two milligrams were incubated with rat liver mitochondria for 2 hours. The same extraction procedure was used as for the pancreatin experiments (p.143). The product was fractionated using reversed phase column chromatography (System M_2).

Result:

	Total counts recovered	
	1 hour	2 hours
7α-Hydroxy ester cholesterol	175	216
% conversion to 7-hydroxy ester	0.5	
% conversion to cholesterol	1.25	

3. Comparison of Oxidation of Cholesterol, Cholesteryl Linoleate and Cholesteryl Stearate

The incubation mixture was the same as that already described except that mouse liver was used and the incubation period was 3 hours.

The extracts were chromatographed as follows (details in the Appendix):-

Silicic acid column - 50% chloroform fraction (cholesterol and 7α-hydroxy-cholesteryl esters)
- 100% chloroform fraction (contains more polar products, 7α-hydroxycholesterol and bile acids).

The 50% chloroform fraction was rechromatographed on a reversed phase column to yield cholesterol and 7α-hydroxycholesteryl esters.

The 100% chloroform fraction was run on the Danielsson Type 3 Column to yield bile acids and the 'trioxyneutral' and 'dioxyneutral' fractions.

Compound	c.p.m. incubated	c.p.m. Recovered				Total % conversion to more polar products
		Cholesterol	7α-OH Cholesterol	Bile acids	Di- & Trioxy products	
Cholesterol, 2 mg.	68,000	Not determined		744 (1%)	[x]26,700 (39%)	40
Cholesteryl stearate, 3 mg.	60,000	96 (0.2%)	106 (0.2%)	325 (0.5%)	410 (0.7%)	1.4
Cholesteryl linoleate, 3 mg.	60,000	175 (0.3%)	527 (0.9%)	420 (0.7%)	710 (1.2%)	2.8

[x] The high activity of this fraction, relative to that for the bile acids, would indicate that considerable autoxidation had occurred. Unfortunately this was not checked.

4. Oxidation of 7α-Hydroxycholesteryl Laurate

(The substrate was tritiated by exposure to tritium after the method of

2 mg. (2.4 μc.) incubated.

Result:

		% Conversion
Recovered as bile acids	0.110 μc.	4.6
Recovered as 'dioxy' and 'trioxy' fraction	0.224 μc.	9.3
	Total	13.9%

CONCLUSIONS

The preliminary nature of these experiments precludes too definite an interpretation of the results. However, some useful information may be derived from them.

It appears that the liver mitochondrial preparation used was not capable of oxidizing cholesteryl esters to any significant extent,

although unesterified cholesterol was efficiently converted to more polar products. Possible reasons for the failure to oxidize the esters are considered in the Discussion.

On the other hand, 7α-hydroxycholesteryl laurate underwent further oxidation relatively easily. This may have been subsequent to hydrolysis to 7α-hydroxycholesterol, but even so the comparison with cholesteryl esters is striking.

DISCUSSION

The work which has been described in Sections 1 and 2 of this Thesis poses two main problems to be solved.

1. How does the presence of linoleic acid in the diet increase the turnover of serum cholesterol?

2. Assuming that the structure for BSM has been correctly assigned as the 3β-linoleyl or oleyl ester of 7α-hydroxycholesterol, what could be the biochemical role of this compound?

It seems possible that the answers to the two problems may be inter-related.

If the turnover of serum cholesterol is increased in the linoleate fed rat, this means that there must be an increase in the rate of appearance of cholesterol in the blood, whether as a result of synthesis or of release from some other source, and also an increase in the rate of removal of cholesterol from the blood. Since it is unlikely that cholesterol is removed merely to be stored elsewhere in the body, it is probable that cholesterol excretion

is accelerated. If this excretion follows the normal pattern, then about 10% would be in the form of neutral sterols, and the remainder as bile acids. There is some evidence that in man the feeding of linoleate, or highly unsaturated oils results in an increased excretion of bile acids (Gordon, Lewis, Eales and Brock, 1957; Haust and Beveridge, 1958; Lewis, 1958), and in an increased excretion of faecal neutral sterols (Hellmann, Rosenfeld, Insull and Ahrens, 1957).

In view of the fact that the feeding of linoleate results in a greater proportion of cholesterol being esterified with linoleate (Mukherjee, Achaya, Deuel and Alfin-Slater, 1958), it seems possible that linoleic acid may exert its effect on cholesterol metabolism by formation of the ester. Up to the present time no information has been published about the metabolism of cholesteryl esters as intact molecules. A considerable volume of work has been reported on the conditions for the formation and hydrolysis of the esters, references to which are quoted in Section 2C, but few speculations have been made as to why cholesterol should be esterified at all.

Some authors have considered that the formation of esters may be a means of transporting either the fatty acid or the cholesterol from one tissue to another (Kinsell, Michaels, Friskey and Splitter, 1959), although it is difficult to see why this should facilitate transport. This theme has been extended by Malkin (1959), who considered that the carriage of esters rather than cholesterol might be easier because the esters, particularly unsaturated ones, have lower melting points. This idea seems irrelevant since although the precise state of cholesterol in the lipoprotein complex is uncertain, it is definitely not carried as a simple oil in water emulsion. In pathological states such as xanthomatosis, large amounts of cholesterol, up to 1,200 mg./100 ml. (the normal level is about 200 mg./100 ml.) are carried in plasma, so that esterification does not seem to be a necessary prerequisite for transport.

It is possible that the formation of esters might allow concentration differences to be established between different points of the cell, or between different organs, but an explanation would still be required for the

subsequent metabolism of the esters so formed.

Inspection of the chemical state in which cholesterol is found in different tissues lends support to the idea that esterification is connected with utilization. In tissues where cholesterol turnover is rapid, the plasma-liver pool and the adrenal gland, a considerable proportion is esterified, from about 20% in liver to about 70% in plasma (Cook, 1956). In those tissues such as brain and nervous tissue, where cholesterol is inert and usually considered to play a structural role, it is in the unesterified state. An interesting corollary to this is that when nerves are damaged and undergo demyelination, the cholesterol disappears from the degenerating myelin, and only at this stage is esterified cholesterol found in nervous tissue (Johnson, McNabb and Rossiter, 1949).

It does seem then that esterification of cholesterol is an important factor in its metabolism. As oxidative transformations, with bile acids as the ultimate stage, are quantitatively the most important reactions undergone by cholesterol, it is interesting to postulate how esterification, particularly with unsaturated fatty acids, may affect these processes.

Bergstrom (1959) has produced evidence that 7-hydroxylation is probably one of the earliest steps in cholesterol degradation, and that the oxygen enters directly from molecular oxygen, with no formation of a ketonic intermediate. He also considers that it may be the rate-limiting step. It is possible that, if the starting compound for this reaction were cholesteryl ester, rather than cholesterol, the oxidation would be more specific. The presence of an ester group at the 3-position favours a selective allylic attack at the 7α-position (Henbest and Jones, 1948). The chemical methods for the preparation of 7-hydroxyl derivatives all use cholesterol esters as starting material (Barr, Heilbron, Parry and Spring, 1936). In contrast, the in vitro oxidation of cholesterol alone results in the formation of 7-oxocholesterol and cholestane-3β-5α-6β-triol as well as the epimeric 7-hydroxyl derivatives (Bergstrom and Wintersteiner, 1941). The studies of Bergstrom and Wintersteiner (1942) showed that esterification of cholesterol produced a very marked inhibition of the aut-oxidation process.

It might be of biological advantage to the organism if the initial step in the breakdown of cholesterol could be made selective.

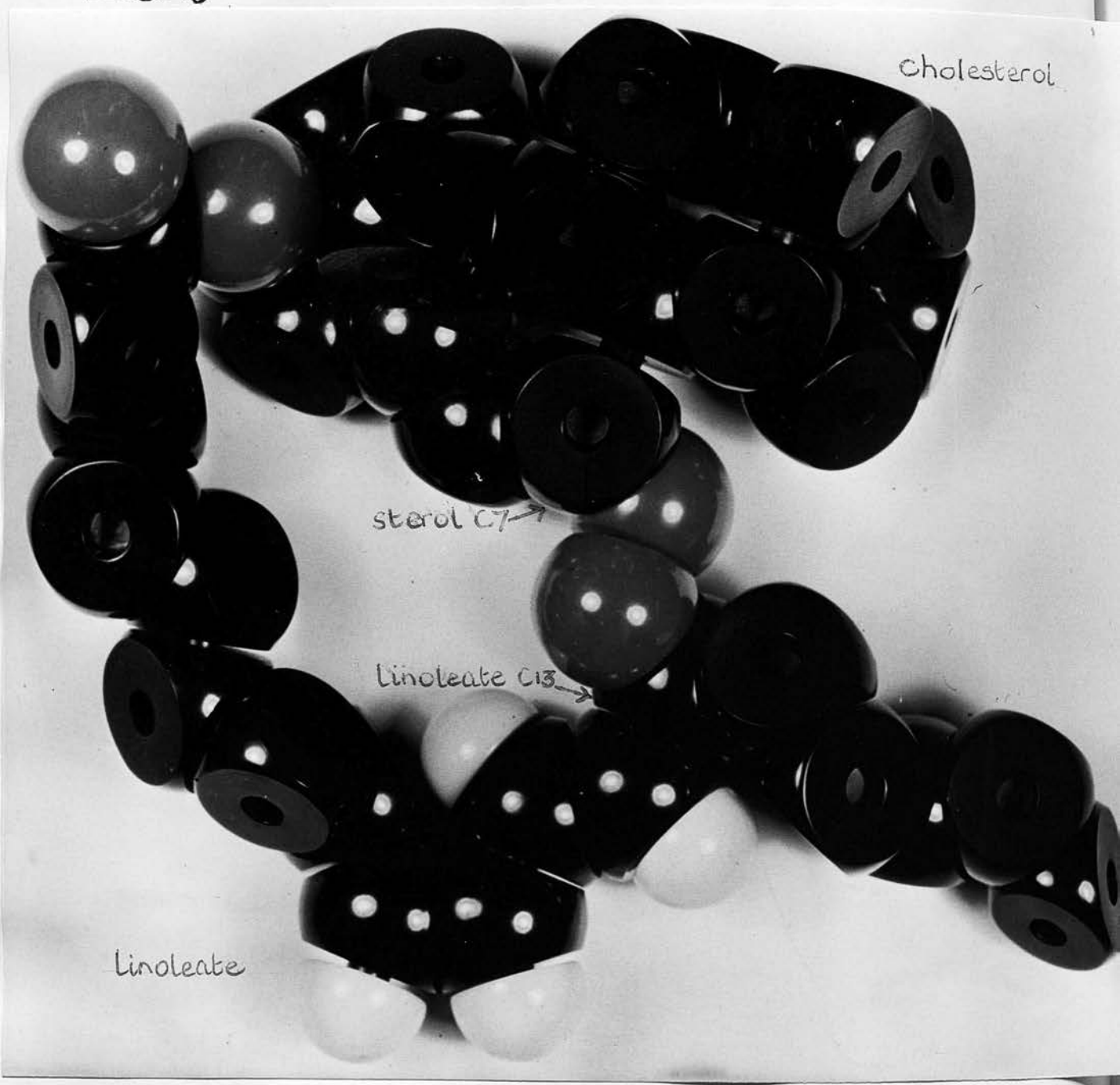
The isolation of BSM from tissues has revealed that cholesteryl esters can exist which have become oxidized at the 7-position of the steroid nucleus. These esters may have arisen by one of two mechanisms, either by oxidation of cholesteryl esters as described above, or by esterification of 7α-hydroxycholesterol. Since the experiments with cholesterol esterase showed that 7α-hydroxycholesterol esters could take part in both the hydrolytic and esterification reactions, it is possible that BSM might occur by direct esterification of 7-hydroxycholesterol. If this were the case, it is difficult to interpret the significance of such a reaction. No unesterified 7-hydroxycholesterol was found in the extracts, which might be expected if esterification had occurred in a similar way to that of cholesterol.

Assuming then that BSM was formed by the first mechanism and that this reaction was of biochemical importance, as an alternative early step in bile acid formation, the question now arises, whether certain cholesteryl esters might be more suitable than others for the promotion

of the reaction. In particular, because of the effect of linoleate on cholesterol turnover, it is of interest to consider how cholesteryl linoleate might behave differently from, say cholesteryl stearate. Of the fatty acids found esterified with cholesterol, only linoleic acid is essential to the rat (Burr and Burr, 1929). Little is known of the role played by linoleic acid, but its deficiency results in features as varied as decreased turnover of serum cholesterol and the inability to maintain water balance (Basnayake and Sinclair, 1956). It can form arachidonic acid by the addition of a two carbon unit, and the formation of extra double bonds (Steinberg, Slaton, Howton and Mead, 1956), and can undergo terminal oxidation (Mead, Slaton and Decker, 1956) though, whether this follows the same route as the saturated acids, is not clear.

The most characteristic reaction in vitro, however, is the peroxidation reaction which has been described in Section 2C (p. 75) and is usually brought about by the plant enzyme lipoxidase.

FIG. 19

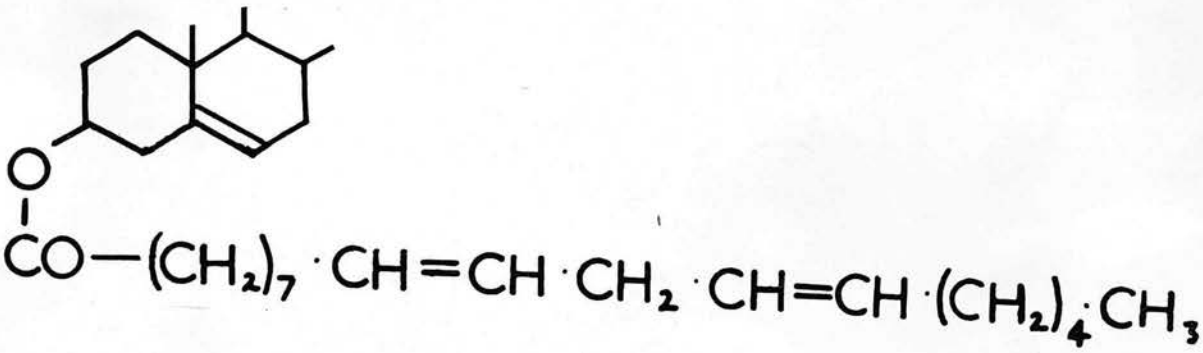


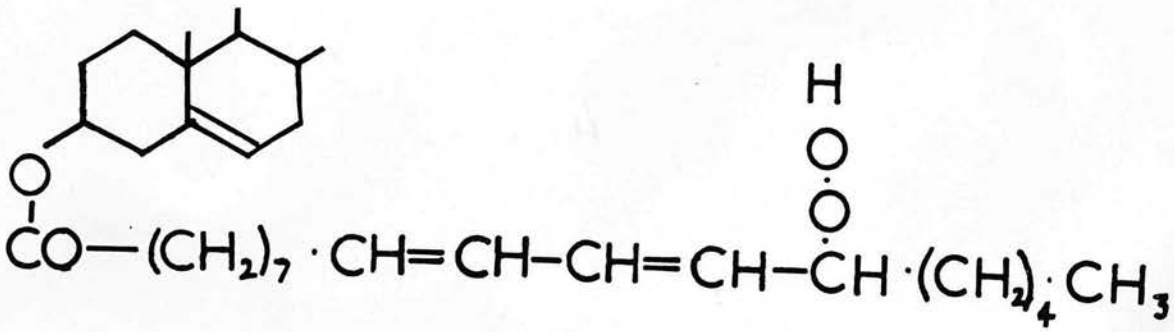
c.f. FIG. 20

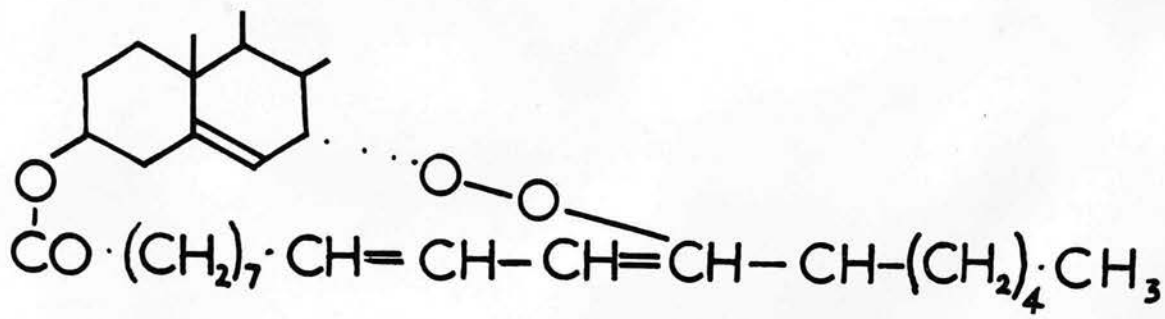
A molecular model of cholesteryl linoleate was made (a photograph of which is seen in Figure 19) and this showed that if a hydroperoxide were formed at the 13-position of the fatty acid and the Δ^9 remained cis while the Δ^{11} became trans, this peroxide would be in juxtaposition to the 7-position of the sterol. Conditions would appear to be suitable then for a linoleate-induced oxidation of the sterol. This might take place by means of a cyclic peroxide, which could then be reduced, perhaps by the agency of a molecule such as TPNH (see Figure 20). In this case however, the resultant molecule would be the ester whose fatty acid was 13-hydroxylinelaidic acid, rather than the simple linoleate.

The reactions described might play an early part in the process of cholesterol oxidation, and Figure 21 shows how they might fit into the accepted scheme. The acyl residue would have to be removed in order to allow epimerization of the 3β-hydroxyl group, but the experiments quoted in Section 2C would indicate that mammalian cholesterol esterase is able to catalyse the hydrolysis of 3-esters of 7-hydroxycholesterol, possibly even more readily than the corresponding

FIG. 20







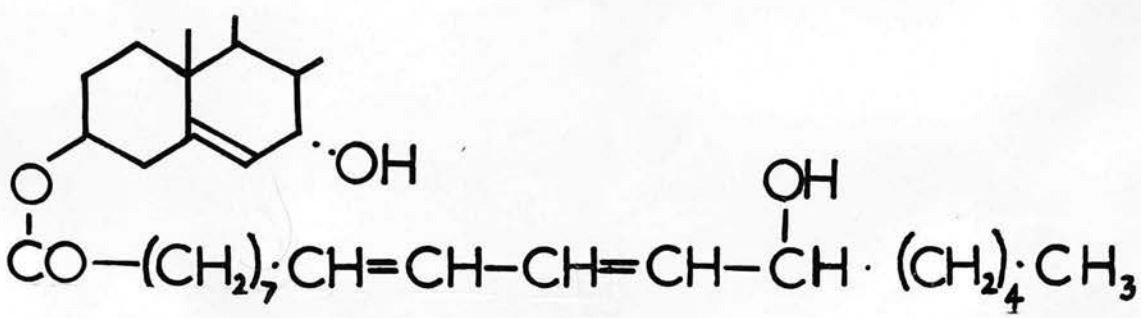
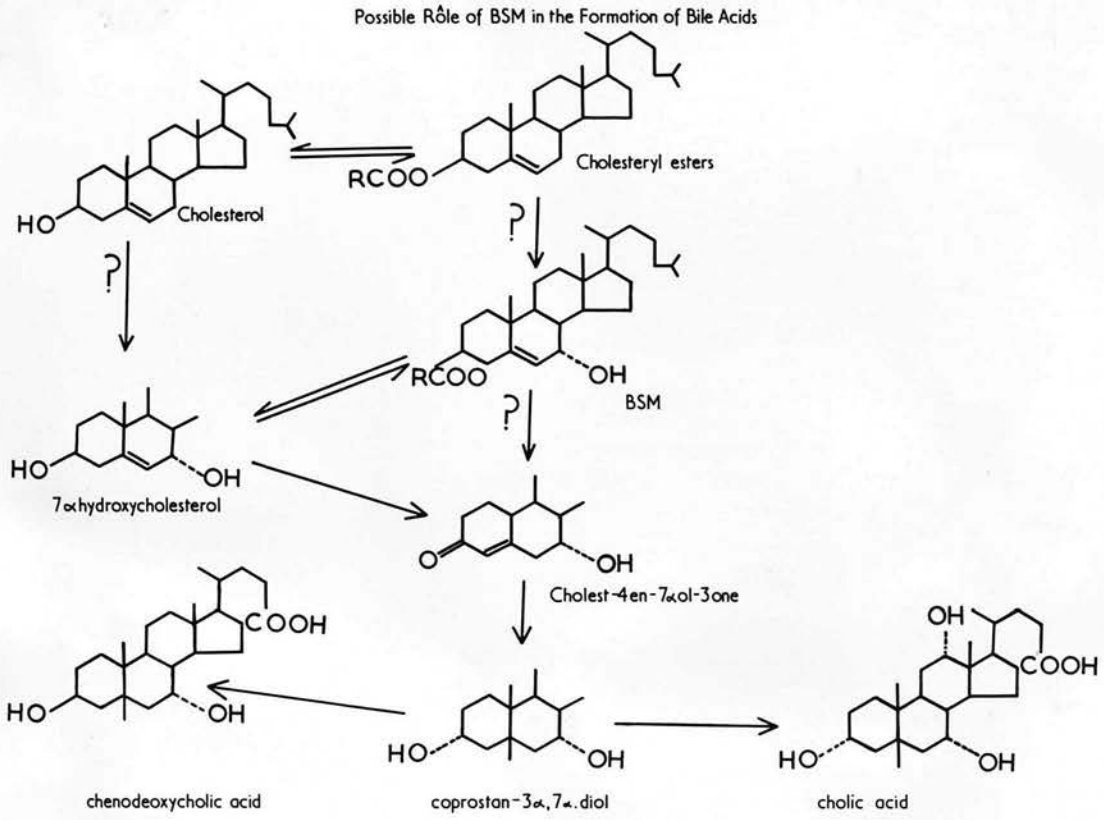


FIG. 21

Possible Rôle of BSM in the Formation of Bile Acids



cholesterol esters. (This fact is of interest since Hernandez and Chaikoff (1957) quote experiments by Korzenovsky, Rust and Diller (1955) which claim that a 7-hydroxyl group prevented pancreatic esterase activity. However, a perusal of the quoted work failed to reveal such a claim. Perhaps the authors were misquoted or the statement was retracted before publication.)

As the epimerization of the 3-hydroxyl group has been shown to involve ketone formation (see p. 41) it is possible that the loss of the acyl residue might be combined with a dehydrogenation reaction, so as to produce Danielsson's intermediate, cholest-4-en-7α-ol-3-one. The types of reaction which could be involved are shown in Figure 21.

The preliminary experiment using tritium labelled 7α-hydroxycholesterol laurate, which was described in Section 2C, indicates that the compound was very readily converted to more polar products. Subsequent experiments might be designed to show whether, or not, this conversion was more rapid than it would be if 7α-hydroxycholesterol were used.

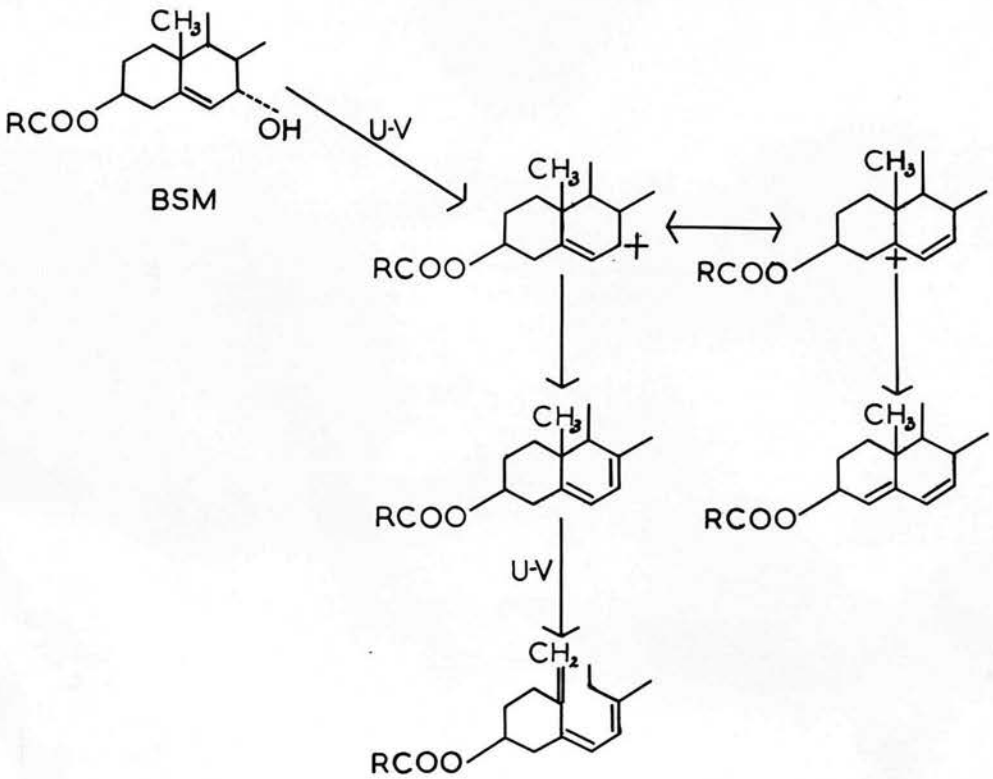
On the other hand, attempts to produce BSM by lipoxidase attack on cholesteryl linoleate

were not successful, although the possibility of a mammalian enzyme with similar activity is not excluded.

The results of preliminary experiments with ^{14}C-labelled substrates, which were designed to compare the ability of cholesteryl esters with that of cholesterol in the formation of bile acids, were not in accord with the scheme which has been proposed. It was found that in the liver mitochondrial oxidizing system cholesterol gave rise to bile acids to far greater extent than did cholesteryl esters, whether saturated or unsaturated.

This apparent failure might be accounted for if the primary oxidation of cholesteryl esters took place, either in a cell fraction other than the mitochondria, or possibly in a different tissue altogether. In view of the fact that BSM was found in leucocytes, it is interesting to speculate whether or not the initial step might take place in the reticulo-endothelial system, and the BSM formed there be transported to the liver for further oxidation. If this did prove to be an alternative pathway of bile acid formation, it would form an interesting parallel to the production of bile pigments.

FIG. 22
Effect of U-V-Light on BSM



A second possible role for BSM is suggested by the reactions of the synthetic esters to ultraviolet light, and the transformation of BSM to u.v. absorbing material on storage (see Figure 22).

A method which has been described for the chemical synthesis of provitamin D utilizes the fact that dehydration at the 7-8-position is facilitated by the presence of a long chain fatty acid residue (Nes, Kostic and Mosettig, 1956).

Vitamin D precursors which are esterified at the 3-position have been isolated from fish liver oils (Hickman and Gray, 1938).

If BSM could function as a precursor of vitamin D ester, the stages involved might be as follows: first, a dehydration, possibly enzymic or u.v. light induced, to give the 5-7-diene, which on absorption of u.v. light gives rise to an open ring structure. The behaviour of the synthetic compounds supports this hypothesis except that the u.v. absorption maximum measured corresponded with a 4-6-diene, rather than a 5-7-diene, and that 7-dehydrocholesterol gives a positive Lifschutz reaction. It is possible that in the chloroform solutions used, there was a shift of double bond from the 5-7 to the 4-6-position.

It is of interest that BSM was found in rat skin, which would certainly be expected if a pro-vitamin D role were a function of the molecule.

Many possibilities present themselves for further experiments on the function of BSM, particularly ones which would establish conclusively how the 7-position becomes oxidized. More quantitative data are required concerning the conversion of BSM to bile acids by comparison with 7α-hydroxycholesterol.

Finally, and most difficult, would be to demonstrate the biological formation of BSM so as to exclude completely the possibility of autoxidation, which always lurks in the background, despite the precautions taken. From this point of view perhaps comfort may be taken in the low yield of BSM, for opinions were expressed nearly twenty years ago that when 7-hydroxy derivatives are obtained from tissues in large amounts they are probably artifacts, but that small amounts are very likely to be genuine intermediates in cholesterol metabolism (Prelog, Ruzicka and Stein, 1943; Haslewood, 1944).

APPENDICES

APPENDIX I

Materials and Methods Used in Work Described in Section 1

MATERIALS

Animals

White male inbred Wistar strain rats were used, whose ages ranged from 54 to 77 days and weights at the end of the experiment from 180 to 260 g. Within any one experiment the ages of the animals did not differ by more than seven days, and litter mates were used when possible.

Foodstuffs

Skimmed milk	-	'Health Stores' dried skimmed milk
Flour	-	Allinsons, London, wholewheat flour
Yeast	-	Distillers Company Ltd., dried yeast extract
Lard	-	Reliable Packing Co., Chicago, iodine no. 65, m.p. 44°C

Sunflower seed oil - T. & H. Smith Ltd., Edinburgh, iodine no. 110, linoleic acid 65.2%, oleic acid 22.8%

Safflower seed oil - Unilever Ltd., linoleic acid 15.5% oleic acid 76.9%

Methyl Stearate

Synthesized by the methanol-sulphuric acid procedure (Deuel, 1951), m.p. 35° (literature 39° loc cit).

Methyl Linoleate

This was obtained as the urea adduct. The ester was released prior to use by cleavage with hydrochloric acid. The adduct was stored at -5°C under nitrogen. (This was generously donated by Unilever Ltd.)

Details of Experiments

Table 1 shows the conditions employed for each experiment.

METHODS

Collection of Blood Samples

The rats were bled by the method described by McGuire (1956). The blood was collected separately from each rat in small specimen tubes, which were then stored at 37°C for half an hour, until clotting was completed. The clots were centrifuged down so that the serum could be removed. A 0.2 ml. sample was measured for each rat, using a blood pipette, and the samples were pooled, either in pairs or per group, according to the experiment.

The serum samples to be pooled were delivered into 7.5 ml. acetone alcohol (1:1 v/v) and refluxed. Aliquots (2 ml.) were removed for cholesterol determination by the method of Sperry and Webb (1950), and 5 ml. aliquots used for ^{14}C determination.

Measurement of Radioactivity of Cholesterol Samples

Each 5 ml. sample (see above) was treated with digitonin and digitonins as in the Sperry-Webb method. The digitonides were dried and cleaved with pyridine, and the

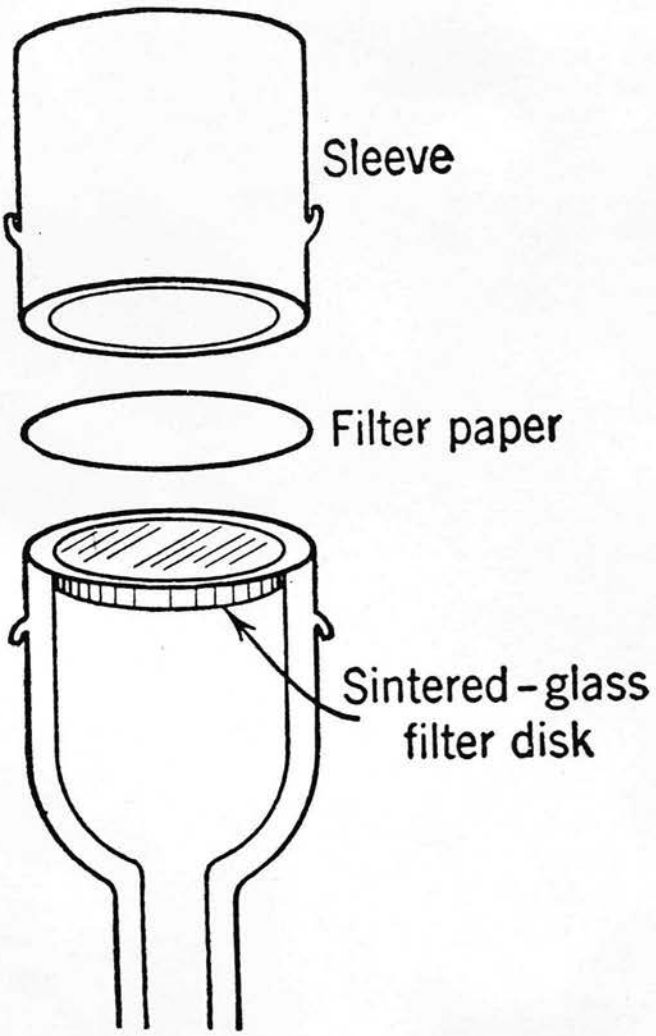


FIG. 23 Glass filter assembly.

free digitonin precipitated by ether (Schoenheimer and Dam , 1933). After removal of this precipitation by centrifugation, the supernatant (which contained the radioactive cholesterol) was taken to dryness, dissolved in 5 ml. of liquid scintillator (N.E. 213, Nuclear Enterprises, Edinburgh) and counted. The apparatus used was an Ecko Scintillation Counter modified to include a water-cooling device which stabilized the temperature at 12°C.

Each sample was counted twice, by determining the time taken to record 1000 counts. Background activity was determined each time the counter was used, and was subtracted from the experimental value.

Collection of Expired $^{14}CO_2$ Samples

The expired $^{14}CO_2$ was trapped in KOH (110 g./l.), using the apparatus shown in Figure 4. The total volume for each one hourly sample was adjusted to 100 ml., then 2 ml. were removed, barium chloride added, and the precipitate filtered off by means of the apparatus shown in Figure 23.

The samples were dried and the films so formed were counted at infinite thickness using an end-window counter.

APPENDIX II

Experimental Results Relating to Work Described in Section 1

Group	Time hr.	Activity of serum cholesterol counts/ min./mg.	Mean Serum cholesterol mg./100 ml.
Experiment I			
Stock	12	2382	53
	60	889	
Linoleate	12	3734	50
	60	845	
Stearate	12	2270	58
	60	998	
Experiment II			
Stock	9	1791	62
	57	697	
Sunflower	9	2996	53
	57	981	

Experimental Results Relating to Work Described in Section 1

Experiment III

Group	Time hr.	Activity of serum cholesterol counts/ min./mg.	Mean serum cholesterol mg./100 ml.
Stock	6	1130	39
	26	1000	
	50	580	
	98	280	
	174	290	
Sunflower	6	2220	46
	26	1620	
	50	715	
	98	202	
	174	85	

Experiment IV

Group	Time hr.	Activity of serum cholesterol counts/ min./mg.	Mean serum cholesterol mg./100 ml.
Stock	6	1458	54
	50	408	
	98	143	
Linoleate	6	1530	51
	50	296	
	98	91	
Stearate	6	1320	59
	50	428	
	98	190	

Experiment VI

Control	Serum cholesterol mg./100 ml.	Cholesterol counts/ min./mg.	Mean activity and standard deviation
15 hr.	69	2340	2492 ± 397
	66	2430	
	73	2140	
	68	3060	
35 hr.	64	2220	1842 ± 297
	67	1890	
	73	1750	
	52	1510	
70 hr.	60	1450	1562 ± 222
	60	1590	
	60	1860	
	56	1350	
92 hr.	67	-	708 ± 156
	64	815	
	64	535	
	58	805	

Experiment VI

Safflower	Serum cholesterol mg./100 ml.	Cholesterol counts/ min./mg.	Mean activity and standard deviation
15 hr.	67	2750	2605 ± 270
	74	2250	
	53	2590	
	66	2830	
35 hr.	66	1880	1481 ± 286
	84	1480	
	62	1355	
	97	1210	
70 hr.	58	810	763 ± 170
	66	905	
	52	575	
	67	-	
92 hr.	67	685	515 ± 202
	76	692	
	70	364	
	69	317	

Experiment VI

Lard	Serum cholesterol mg./100 ml.	Cholesterol counts/ min./mg.	Mean activity and standard deviation
15 hr.	60	3690	3055 ± 597
	64	3310	
	64	2930	
	63	2290	
35 hr.	59	2930	2462 ± 438
	51	2720	
	59	1980	
	69	2220	
70 hr.	59	1695	1460 ± 146
	54	1440	
	59	1410	
	68	1295	
92 hr.	59	1145	979 ± 142
	64	1070	
	61	772	
	68	930	

Summary of Results

Experiment		Half-Life serum cholesterol (hours)	Rate of Synthesis (as percentage of control)
I	Stock	36	100
	Linoleate	20	193
	Stearate	44	91
II	Stock	36	100
	Sunflower	30	163
III	Stock	44	100
	Sunflower	27	186
IV	Stock	28	100
	Linoleate	18	126
	Stearate	28	93
V	Stock	not determined	not determined
	Safflower	31	not determined
	Lard	48	not determined

Note: The Control Group in Experiment VI produced results too scattered for a useful line to be drawn. This group was therefore not considered in the statistical analysis.

TABLE 9

SPECIFICITY OF LIFSCHUTZ REACTION

Compound	+ or -	Notes
Cholesterol	-	
Cholesteryl esters	-	
Cholest-4-en-3-one	-	
Cholest-5-en-3-one	-	
Cholestan-3-one	-	
Coprostan-3-one	-	
Cholest-5-en-7-one	-	
3β-Acetoxycholest-5-en-7-one	-	
Cholestan-3β-ol	-	
Coprostan-3β-ol	-	
Cholestan-3α-ol	-	
Coprostan-3α-ol	-	
Cholest-5-en-3β-7α-diol	+	normal reaction
3β-Acyloxy-cholest-5-en-7α-ol	+	normal reaction
3β-Acyloxy-cholest-6-en-5α-ol	+	intense reaction
Cholest-4-en-3-one-7α-ol	-	
Cholest-5:7-diene	+	
Cholest-3:5-diene-7-one	-	
Cholest-4:6-diene-3-one	-	
Vitamin A	-	
Vitamin A palmitate	+	very rapid, fleeting colour
3α-7α-dihydroxy-coprostane	-	
3α-7α-12α-trihydroxy-coprostane	-	
Cholic acid	-	
Chenodesoxycholic acid	-	

APPENDIX III

Details of Chromatographic Procedures Used in the Work Described in Section II

1. Lifschütz Colour Reaction

The preparation of the reagent (90 ml. of glacial acetic acid, 10 ml. of concentrated sulphuric acid and 0.1 g. of ferric chloride and water) and the reaction conditions were identical with those described by Bergstrom and Wintersteiner (1942).

The relationship between light absorption at 640 mμ and concentration was linear within the range of 20-100 μg. chromogen. At higher concentrations, proportionately more colour was produced, and so aliquots taken for estimation were always calculated to come within the above range. Calibration determinations were performed with each estimation, as the reagent altered slightly on storage and a permanent calibration curve was not reliable.

Specificity of the Reaction

Table 9 summarizes the findings.

TABLE 10

SPECIFICITY OF PHOSPHOTUNGSTIC ACID REACTION

Compound	Reaction	Time for colour to appear
Cholesterol	pink	15 sec. hot plate
Cholesteryl esters	pink	15 sec. hot plate
Cholest-4-en-3-one	none	
Cholest-5-en-3-one	pink	15 sec. hot plate
Cholestan-3-one	none	
Coprostan-3-one	none	
Cholest-5-en-7-one	none	
3β-Acetoxy-cholest-5-en-7-one	none	
Cholestan-3β-ol	pink	1 hr. at 37°
Coprostan-3β-ol	pink	1 hr. at 37°
Cholestan-3α-ol	pink	1 hr. at 37°
Coprostan-3α-ol	pink	1 hr. at 37°
Cholest-5-en-3β:7α-diol	blue	10 sec. hot plate
3β-Acyl-cholest-5-en-7α-ol	blue	10 sec. hot plate
3β-Acyl-cholest-6-en-5α-ol	blue	10 sec. hot plate
Cholest-4-en-3-one-7α-ol	pink	10 sec. hot plate
3β-Stearoxy-cholest-5:7-diene	blue	10 sec. hot plate
Cholest-3:5-diene-7-one	none	
Cholest-4:6-diene-3-one	none	
Vitamin A	none	
Vitamin A palmitate	bluish-grey	30 sec. - faded quickly
3α-7α-Dihydroxy-coprostane	none	
Cholic acid	none	

2. Phosphotungstic Acid Staining Reaction

The phosphotungstic acid was dissolved in ethanol, as described by Martin (1957), who recommended a 15% (w/v) solution. However, a 10% solution was found more satisfactory as crystallization occurred in the capillary of the chromatography spray when the higher concentration was used. The papers were sprayed very lightly and care was taken to avoid high local concentrations of reagent as this caused scorching. The papers were dried by moving them gently at a height of about 6 inches above an electric hot plate set at 'medium'. The colour of the spots changed on heating in some cases. Details of the compounds tested with this reagent are given in Table 10. For the purpose of the tests, approximately 10 μg. were sprayed over an area of 1 cm^2.

3. Paper Chromatography System

The paper used (Whatman no. 3) was cut into pieces, 6" x 20", taking care that the grain of the paper ran vertically. The papers were impregnated with liquid paraffin immediately before use by immersing them in a solution of 10% paraffin in petroleum ether. The solution was placed in a flat glass dish and the papers drawn through rapidly six times. They were then blotted gently between sheets of blotting paper by means of a photographic roller. It was important that the impregnation procedure should be standardized as the concentration of paraffin had a marked effect on the mobility of compounds separated. As a check, standard compounds were run in each experiment.

The papers were allowed to dry at room temperature before being 'spotted' with compounds. Spots were applied by means of individual capillary tubes for qualitative work, and a micrometer syringe when carefully measured aliquots were required.

The mobile phase consisted of chloroform, methanol and paraffin (40/50/10 v/v/v), which were well shaken, and then left to equilibrate;

TABLE 11

MOBILITY OF STANDARD COMPOUNDS IN THE PAPER SYSTEM

Compound	R_F	Method of Detection
Cholesterol (cholest-5-en-3β-ol)	0.70	PTA
Cholesteryl myristate	0.15	PTA
7α-Hydroxycholesterol (cholest-5-en-3β-7α-diol)	0.88	PTA
7α-Hydroxycholesterol 3β-myristate	0.51	PTA
Vitamin A	0.85	yellow colour u.v. absorption
Vitamin A palmitate	0.29	PTA u.v. absorption
Cholest-5:7-diene	0.33	u.v. absorption
Cholest-3:5-dien-7-one	0.60	u.v. absorption
Cholest-4:6-dien-3-one	0.67	u.v. absorption
Cholest-5-en-7-one-3β-ol	0.87	u.v. absorption

the lower phase was used to develop the chromatograms.

Descending chromatography was used; the papers were placed in troughs within tanks which were saturated with the mobile phase, and allowed to equilibrate for a minimum period of four hours. Chromatography was started by introducing the mobile phase into the troughs. The papers were removed when the solvent front had run for about three-quarters of the length of the paper, which normally took about sixteen hours.

The papers were dried at room temperature, examined in ultraviolet light, and any spots visible were noted. They were then sprayed with the phosphotungstic acid reagent.

R_F's of standard compounds are given in Table 11.

Details of the R_F's of different esters of 7α-hydroxycholesterol are given in Table 4 (p. 50) and are not repeated here.

TABLE 12

ELUTION CHARACTERISTICS

OF SILICIC ACID COLUMN

mobile phase (petroleum ether 60-80°
(chloroform

Percentage Composition of Mobile Phase		Volume	Material Eluted
Petroleum Ether	Chloroform	ml.	
100	0	50	hydrocarbons-carotenes
99	1	100	cholesteryl esters
90	10	50	triglycerides
75	25	50	none detectable
50	50	200	cholesterol vitamin A esters
40	60	50	none detectable
25	75	100	7α-hydroxy-cholesterol
0	100	200	cholic acid chenodesoxy-cholic acid

The volumes quoted are for a 25 g. column.

4. Silicic Acid Column Chromatography

The method adopted was modified from that described by Ahrens and Hirsch (1958). The silicic acid was prepared exactly according to their instructions, but the solvent system was altered because it was considered desirable to exclude ether, and consequently a system consisting of petroleum ether, 60-80°, and chloroform was adopted. The size of column used depended upon the weight of material to be resolved, but in general 20-25 g. were used, forming a column 1.8 cm. x 12 cm., and the lipid extract from 20 ml. of serum could be placed on this. In order to achieve a satisfactory flow rate, the columns were normally run under a pressure of approximately 70 mm.

The elution pattern of this type of column is seen in Table 12.

Note: In the isotopic experiments (p. 101) where there is reference to '100% chloroform cuts', no 75% chloroform cuts were taken. Hence the 100% cuts contained 7α-hydroxycholesterol as well as bile acids.

TABLE 13

REVERSED PHASE SYSTEMS USED

Designation	Mobile Phase isopropanol ml.	Mobile Phase water ml.	Stationary Phase chloroform ml.	Stationary Phase heptane ml.
Danielsson Phase System I	165	135	10	40
Danielsson Phase System III	150	150	15	60
System M_2 (Mawer)	200	100	5	45

5. Reversed Phase Column Chromatography

The method used was that of Danielsson (1958) but, in addition, a modification of it was used in order to produce separations which could not be achieved with Danielsson's system.

The columns consisted of Hyflo Supercel (Johns Manville) which had been exposed to dichlorodimethylsilane to render it hydrophobic. The celite was mixed with the stationary phase, and then slurried with mobile phase as described by Danielsson. The proportions were 3 g. of celite to 2.7 ml. of stationary phase.

Columns 250 mm. x 13 mm. internal diameter were used and 3 ml. fractions were collected using a Locarte automatic fraction collector. Solvents were equilibrated and the columns run at a constant temperature of 25°C.

The contents of the fractions were identified by removing the solvent, either in a warm oven or in a blast of nitrogen, and testing by means of a Liebermann-Burchard or Lifschütz colour reaction, or by ultraviolet absorption.

Data for the columns are collected in Tables 13 and 14.

TABLE 14

ELUTION CHARACTERISTICS OF STANDARD COMPOUNDS IN THE REVERSED PHASE COLUMN SYSTEMS

(1-3 mg. each compound per column)

Compound	Volume of mobile phase required for elution (ml.)		
	System D III	System D I	System M_2
3α:7α-Dihydroxy-cholanic acid (chenodeoxycholic)	7-15	eluted immediately	eluted immediately
3α:7α:12α-Tri-hydroxy cholanic acid (cholic)	16-24		
Cholest-5-en-3β-7α-diol	75-100	24-29	15-18
Cholest-5-en-3β-ol	not eluted with 200 ml.	60-70	20-27
3β-Acetoxycholest-5-en-7α-ol		90-110	30-45
3β-Palmitoxy-cholest-5-en-7α-ol		140-180	80-95
Vitamin A palmitate		not eluted	140-150
[x] 3α:7α:12α-Tri-hydroxy coprostane	50-65	15-25	-
[x] 3α:7α-Dihydroxy coprostane	110-130	35-45	-

[x] These figures were not determined, but are quoted from Danielsson (1958).

Application of the Silicic Acid and Reversed Phase Column Systems

The silicic acid columns were used to separate cholesterol from cholesteryl esters when lipid extracts of tissues were investigated. The 50% chloroform fraction contained other compounds, notably the esters of 7α-hydroxycholesterol, and resolution was possible on a reversed phase column, type M_2. The Danielsson type I column was not suitable as the esters were eluted too late and spread over too large a volume.

In the quantitative experiments involving esterification and hydrolysis of 7α-hydroxycholesterol, small silicic acid columns (5 g.) were used. Separation of the alcohol and its esters was possible, by taking the 50% chloroform fraction for the esters, running through some 60% chloroform, and then collecting the 75% chloroform fraction which contained the free alcohol. The 7α-hydroxycholesterol was sometimes eluted with 100% chloroform so as to obtain it in a smaller volume of solvent.

Both types of column were utilized in the isotopic experiments described in Section 2C. The extracts were separated first on a silicic acid column to give a '50% chloroform cut', which contained cholesterol and 7α-hydroxy-cholesteryl esters, when the latter were present. After running through some 60% chloroform, the remaining polar material was eluted together, consisting of bile acids, 7α-hydroxycholesterol and presumably also the di- and trihydroxy coprostanes. Because of the preliminary nature of the isotopic experiments, methods of detecting these last compounds were not developed, but since their polarity is comparable with that of 7α-hydroxycholesterol it seems likely that they would be eluted in the 100% chloroform cut. No radioactivity was detectable in the 60% chloroform cut.

The 50% chloroform cut was run on a Type M_2 column to separate cholesterol from esterified 7α-hydroxycholesterol. The 100% cut was run on a Type D III column to obtain the bile acids and the polar neutral sterols in separate fractions. It was considered that the data quoted by Danielsson for the hydroxycoprostanes

was applicable since his elution patterns had been reproduced exactly as far as the other compounds were concerned.

Hence four fractions were obtained for determination of radioactivity. The ^{14}C estimations were performed in the manner described in Appendix I (p. 121). Tritium determinations were kindly performed by the Medical Physics Unit of Edinburgh University.

APPENDIX IV

1. Sources of Materials Used in Chromatographic Procedures

Phosphotungstic acid	B.D.H. Lab. reagent
Liquid paraffin	B.D.H. 0.83-0.87 density
Petroleum ether	J.F. Macfarlan & Co., Edinburgh (60°-80°)
Chloroform	T. & H. Smith Ltd., Edinburgh.
Silicic acid	Mallinkrodt, St. Louis, U.S.A. (100 mesh)
Hyflo supercel	Johns Manville & Co.
Dichloro-dimethyl-silane	B.D.H. Lab. reagent
Isopropanol	J.F. Macfarlan & Co., Edinburgh.

2. Sources of Chromatographic Standards

A. Obtained from external sources

Compound	Source
Cholesterol	Weddel Ltd. Analytical sample purified by bromination procedure (Fieser, 1953)
Cholestan-3-one Cholestan-3β-ol	L. Light Ltd.
Cholestan-3α-ol	Collection of Dr W. Klyne
Cholest-5:7-diene-3β-ol	
Vitamin A Vitamin A palmitate	Glaxo Ltd.
Cholic acid Chenodeoxycholic acid 3α:7α-Dihydroxy-coprostane 3α:7α:12α-Trihydroxy-coprostane Cholest-4-en-7α-ol-3-one	Collection of Dr G.S. Boyd

All the above compounds compared satisfactorily with the reported criteria of purity.

B. Compounds synthesized by the author

Compound	Characteristics					Reference
	m.p. °C		u.v. abs. max.			
	Theory	Observed	mμ	Theory	Observed	
Cholest-4-en-3-one	82	81	240	18,000	17,800	Fieser (1955)
Cholest-5-en-3-one	124	120-122				Djerassi, Engle and Bowers (1956)
Coprostan-3-one	63	63				Grasshof (1934)
Coprostan-3β-ol	101	97-98				Ruzicka (1934)
Coprostan-3α-ol	117	118-120				Ruzicka (1942)
Cholest-5-en-3β-ol-7-one	170	170	235	14,000	14,400	Fieser, Fieser and Chakravarti (1949)
3β-Acetoxy-cholest-5-en-7-one	156-158	157-159				
Cholest-3:5-dien-7-one	110	108-109	278	24,400	27,000	Stavely and Bergmann (1937)
Cholest-4:6-dien-3-one	82	79-81	284	25,000	27,000	Mandell (1956)
Cholest-5-en-3β-7α-diol	[x]184	178-180				Barr, Heilbron, Parry and Spring (1936)

The syntheses of cholesteryl esters and of 3β-esters of 7α-hydroxycholesterol have been described in Section 2B of this Thesis.

[x] The melting point obtained varies with the amount of methanol of crystallization; data recorded vary by as much as 20°. The sample synthesized did not depress the melting point of a sample of 7α-hydroxycholesterol obtained from Professor Wintersteiner.

APPENDIX V

Extraction Procedures Used in the Quantitative Enzymic Experiments Involving 7α-Hydroxycholesterol and its Esters

1. Outline of the Procedure

Pyrex boiling tubes (50 ml.) fitted with ground glass stoppers and sockets were used as vessels for the incubation mixtures. The tubes were incubated, unstoppered, in a water bath at 37°C for the required time, when the reaction was stopped by the addition of 20 ml. of ethanol/acetone (1:1 v/v). Condensers were fitted to the tubes which were heated until the solvent refluxed. After cooling, the tubes were centrifuged, and the supernatants carefully decanted into 100 ml. round-bottomed flasks. The residues were extracted twice with 20 ml. portions of ethyl acetate, and the supernatants added to the round-bottomed flasks. Solvents were removed by suction, and the residues were treated with small volumes of petroleum ether 60-80% chloroform (4:1 v/v) until the lipid had been extracted.

This was necessary as a considerable amount of non-lipid residue, from the pancreatin, was extracted by the ethanol/acetone. The non-lipid material was discarded by removing the petroleum ether/chloroform solution through a pipette fitted with a cotton wool plug. Four extractions with 2 ml. portions of petroleum ether/chloroform were sufficient. These extracts were placed on small silicic acid columns (5 g. of silicic acid) which had been prepared with the appropriate solvent mixture. Petroleum ether alone did not extract the sterol efficiently, and as the earliest fractions to be collected from the column were those eluted with petroleum ether/chloroform (1:1 v/v) the presence of 20% chloroform in the initial fractions was justified.

Elution was as follows:-

25 ml.	petroleum ether	80%	chloroform	20%
100 ml.	"	"	50%	"	50%
25 ml.	"	"	40%	"	60%
100 ml.	"	"	0%	"	100%

Check experiments showed that with weights of up to 2 mg. of sterol, all the 7α-hydroxy-cholesterol esters were contained in the 50% chloroform fraction and all the free alcohol in the 100%.

These fractions were taken to dryness and then dissolved in a given volume of chloroform so that 1 ml. of solution would contain approximately 100 μg. of chromogen. Duplicate 1 ml. portions were then estimated by the Lifschütz colour reaction.

2. Choice of Solvent for the Extraction Procedure

The following solvents were tried, alone, and in various combinations, but satisfactory recoveries were not achieved:

ethanol, methanol, methylal, chloroform.

The effect of ether on 7α-hydroxycholesterol was most unexpected. A low Lifschütz reading was given, and the characteristic blue-green colour did not develop fully but gave way to a red colour on standing. This absorbed maximally at 500 mμ wavelength instead of the normal 640 mμ. The point at which the compound became liable to destruction by ether was during the evaporation to dryness by suction. This was more marked at low concentrations of sterol, so presumably it was attacked when in a film on the walls of the flask. Treatment of the ether with ferrous

sulphate to remove peroxides increased the recovery a little, but never above 60%.

The effect of ether on different concentrations of sterol was investigated. The solutions were sucked to dryness, the residues dissolved in chloroform, and a volume removed to contain 100 µg. of chromogen if recovery were 100%.

Control solutions of chloroform were treated similarly.

Results

Solvent	Total weight of 7α-hydroxycholesterol per flask (µg.)	Optical density of theoretical 100 µg. 640 mµ	500 mµ
Ether	100	0.170	0.400
	200	0.225	0.295
	300	0.311	0.252
Chloroform	100	0.700	0.140
	200	0.741	0.145
	300	0.720	0.140

Only when ethyl acetate was used could recoveries of about 90% be obtained.

3. Analytical Results of Recovery Experiments

a) Effect of applying extract to column in petroleum ether/chloroform 4:1 v/v

Contents of Tube	Optical Density
80 µg. Standards	0.506
	0.516
100% Chloroform cuts from column where extract applied in petroleum ether/ chloroform	0.470
100% Chloroform cuts from column where extract applied in petroleum ether	0.216
	0.209

b) Overall recovery of 7α-hydroxycholesterol

100 μg. added to the mixture.

Final volume of extract taken to contain 40 μg.

Tube	Optical Density at 640 mμ	
Standard	0.230	
1	0.195	
2	0.200	
3	0.230	Mean Percentage Recovery
4	0.219	
5	0.195	91 ± 4
6	0.220	
7	0.197	
8	0.207	

c) Overall recovery of 7α-hydroxycholesterol laurate

150 μg. of sterol; volume for determination contained 75 μg.

Tube	Optical Density at 640 mμ	
Standard	0.568	
1	0.510	
2	0.498	
3	0.500	Mean Percentage Recovery
4	0.506	
5	0.507	96 ± 1
6	0.500	
7	0.520	
8	0.497	

S U M M A R Y

1. Experiments are described whose results indicate that the half-life of serum ^{14}C-labelled cholesterol in the rat was decreased when the animals had been pre-fed a diet containing unsaturated fat.

2. In most of these experiments there was also an indication that the rate of biosynthesis of cholesterol from acetate was increased when the animals had been pre-fed such a diet.

3. A proposed explanation for these phenomena is that unsaturated fatty acids may combine with cholesterol to form esters which are metabolized more readily than are esters formed with other fatty acids.

4. The isolation of a new Lifschütz chromogen is described. The material was present in human serum and leucocytes and in rat serum, skin and liver. It was shown to be identical with the 3β-oleyl and linoleyl esters of 7α-hydroxycholesterol.

5. The chemical synthesis of a series of 3β-acyl esters of 7α-hydroxycholesterol is described in detail, as previously a method

has been reported only for the acetyl ester.

The physical and chromatographic properties of the esters are tabulated.

6. The synthetic esters were used as substrates for enzymic experiments. The results of these experiments show that the cholesterol esterase system of pancreas was effective for both the hydrolysis and synthesis of the esters, and that of liver for hydrolysis only.

7. The esters were efficiently metabolized to bile acids *in vitro*.

8. Attempts to produce the synthetic esters either by enzymic oxidation or by autoxidation of cholesteryl esters were not successful.

9. A description is given of the curious changes undergone by the esters on exposure to ultraviolet light. The resultant changes in structure are discussed.

10. A possible rôle for the naturally occurring esters is suggested, whereby they may form an alternative pathway in the production of bile acids from cholesterol.

11. A hypothesis is described in which it is proposed that the preferential oxidation of certain cholesteryl esters to form esters of 7α-hydroxycholesterol may explain the effect of unsaturated fatty acids on cholesterol turnover.

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A Method for the Specific Differential Estimation of Cholic and Chenodeoxycholic Acids in Human Plasma. By R. H. P. Reid and G. S. Boyd. (*Department of Biochemistry, University of Edinburgh*)

A method has been developed for the estimation of these two acids in plasma which involves the chromatographic separation of these acids followed by spectrophotometric analysis by a modified Pettenkoffer reaction. It is admirably suited for estimations on certain pathological plasma samples and can be modified for use on normal plasma. The procedure for plasma from jaundiced subjects is as follows: 2·0 ml. of plasma is extracted twice with 25 ml. ethanol and the ethanolic extracts are pooled and evaporated to dryness. The residue is dissolved in 25 ml. 0·1 M-$NaHCO_3$–Na_2CO_3 buffer and extracted with an equal volume of light petroleum (60–80°). The aqueous phase is transferred to a nickel crucible using 25 ml. 5N-NaOH in the process, the crucible is covered, and autoclaved at 110° for 3 hr. After cooling, the solution is made acidic to litmus by the addition of concentrated hydrochloric acid and extracted with 3 × 70 ml. chloroform–ethanol (9:1). The extracts are taken to dryness *in vacuo* and the residue transferred to a boiling tube with ethanol. The ethanolic solution is evaporated to dryness and dissolved in 0·75 ml. 70 % acetic acid. This solution is absorbed into 0·75 g. celite, slurried in light petroleum (60–80°) and packed on the top of a Celite column (4 g., height 4·5 in.) employing the partition system described by Matschiner *et al.* (1957). The column is then gradient eluted with successively increasing amounts of benzene (6 gradient steps with 20 % increments—each step volume of 25 ml.). Cuts of volume 10·0 ml. are taken and evaporated to dryness by heating in an oven for at least 8 hr. at about 70°. The cuts are then analysed by addition of 5·0 ml. of 70 % H_2SO_4 followed by 1·0 ml. of 0·25 % furfuraldehyde. After 70 min. at room temperature the pink colour is measured at 5100 Å and the concentrations of the two acids calculated by comparison with the appropriate calibration graphs. This sensitive modified Pettenkofer reaction used in conjunction with the rest of this procedure appears to be specific for cholic acid and chenodeoxycholic acids. The colour is represented by a sharp absorption maximum at 5100 Å.

Cholic and chenodeoxycholic acids are completely separated on these columns and the background reading over the entire range of cuts is consistently low. Recovery experiments on 100 μg. sodium taurocholate give recoveries of 75 % ± 4 %. This is also the efficiency of the procedure when applied to pure solutions.

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The Effect of Dietary Linoleic Acid on Serum Cholesterol in the Rat. By G. S. Boyd and E. Barbara Mawer. (*Department of Biochemistry, University of Edinburgh*)

The feeding of linoleic acid or oils rich in this acid to rats has been shown to lower serum cholesterol below that of rats on a saturated fat diet (Avigan & Steinberg, 1958). An increased rate of synthesis of cholesterol by livers of linoleate-fed rats has been reported by Merrill (1958) and Mukherjee & Alfin-Slater (1958). The present work was undertaken to investigate the effect of dietary linoleate on the serum cholesterol of the rat.

White male Wistar inbred rats were fed a stock low-fat diet: 70 % wholemeal flour, 25 % dried skimmed milk, 5 % dried yeast. After 7 days the animals were divided into three groups: (1) controls on stock diet, (2) 10 % calories replaced by methyl linoleate, (3) 10 % calories as methyl stearate. After 8 days on the diets each animal was injected i.p. with 30 μc [1-^{14}C]acetate (100 μmoles). Over the next 100 hr. blood samples were withdrawn from the tail and the specific activity of the serum cholesterol determined. From a plot of the log of the specific activity against time, the rate of incorporation of ^{14}C into cholesterol at zero time was calculated for each group and used as an index of the rate of synthesis of serum cholesterol.

The methyl linoleate feeding resulted in the 'half life' of the serum cholesterol being decreased to 60 % of the control value and the rate of synthesis increased to 160 % of the control. On feeding methyl stearate the converse was observed, the 'half life' being increased and the rate of synthesis decreased. The experiment was repeated using sunflower seed oil (iodine value 110), which gave similar results to linoleate feeding. The serum cholesterol levels of linoleate-fed animals were lower than those of stearate-fed or of the control animals. In all cases the animals grew normally

and showed no signs of essential fatty acid deficiency.

It is suggested that linoleic acid may act as a cofactor in the metabolism of cholesterol in the rat, its presence increasing the rate of turnover of serum cholesterol.

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Preliminary Observations on the Incorporation of [2-^{14}C]Acetate and [2-^{14}C]Mevalonate into the Unsaponifiable Matter of Isolated Chromatophores of *Rhodospirillum rubrum*. By B. H. Davies and T. W. Goodwin. (*Department of Biochemistry, The University of Liverpool, Liverpool* 3)

Spirilloxanthin, the major carotenoid pigment of *Rhodospirillum rubrum*, is found exclusively in the chromatophores. It is accompanied in the unsaponifiable fraction of chromatophores by a highly polar material which is strongly adsorbed on alumina (Land, 1955) and which gives an R_F of 0·72 in reversed phase paper chromatography using solvent 'C' of Kodicek & Ashby (1954). The spirilloxanthin fraction is relatively more strongly labelled than this compound (which gives none of the characteristic sterol tests) when [2-^{14}C]mevalonate is used than when [2-^{14}C]acetate is used.

[2-^{14}C]Acetate is completely metabolized within 24 hr. by growing cells of *R. rubrum*, incubated anaerobically in the light, and 6–7 % of the total activity is found in the unsaponifiable fraction. On the other hand, under the same conditions, [2-^{14}C]mevalonate is insignificantly metabolized, only about 0·10–0·20 % being found in the unsaponifiable fraction.

Chromatophores prepared by ultrasonic disintegration were resuspended in M/15 phosphate buffer (pH 7·0) anaerobically in the light for 4 hr. in the presence of the labelled substrate. [2-^{14}C]-Acetate was incorporated to the extent of about 0·2 %, and [2-^{14}C]mevalonate to about 0·03 %. Thus in isolated chromatophores, incorporation of acetate is reduced to a much greater extent than is mevalonate incorporation. Attempts to increase the uptake of both acetate and mevalonate by isolated chromatophores have been made by adding (*a*) co-factors (e.g. ATP, TPN and Mn^{2+}), (*b*) a metabolizable substrate (e.g. malate), and (*c*) the supernatant fraction from disintegrated cells. No significant improvement has yet been achieved.

The [2-^{14}C]mevalonate was kindly supplied by Hoffmann-La Roche and Co. (Dr O. Isler); B.H.D. is in receipt of an A.R.C. Research Studentship.

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Non-esterified Fatty Acids in Sheep Plasma. By D. B. Lindsay. (*A.R.C. Institute of Animal Physiology, Babraham, Cambridge*)

In man (see Frederickson & Gordon, 1958) the non-esterified fatty acids (NEFA) found in plasma are mainly of chain length greater than C_{14} and probably arise from adipose tissue. The plasma level rises on fasting overnight, quickly falling after taking a meal or glucose.

In ruminants, the presence of volatile fatty acids in plasma is well known. But as little information is available concerning the long chain NEFA this has been investigated. It has been found that in the sheep little change is observed in plasma NEFA after a 24 hr. fast. If, however, the rumen is first emptied, a fast of 24 hr. regularly produces a rise in plasma NEFA. The level falls within an hour or two of putting fresh contents in the rumen; and almost immediately if glucose or acetate is infused intravenously.

When acetate was infused intravenously, and interference with glucose utilization was induced in some way, the plasma concentrations of NEFA and acetate increased. This was observed either by giving insulin to bring the blood sugar below 20 mg./100 ml., by giving adrenaline, or by producing alloxan diabetes.

The metabolism of NEFA, acetate and glucose would appear to be interrelated: this may be because (*a*) glucose is required for the oxidation of both long- and short-chain fatty acids; or (*b*) in the presence of glucose, acetate is used for fat synthesis in adipose tissue and if fat synthesis is reduced, long-chain fatty acids are released.

Experiments with ^{14}C-labelled acetate are being carried out to distinguish between these possibilities.

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The Identification of Lifschütz-Positive Material Appearing in the Cholesterol Fraction of Lipid Extracts

By G. S. Boyd and E. Barbara Mawer. (*Department of Biochemistry, University of Edinburgh*)

Lipid extracts, made under strictly anaerobic conditions, from a variety of mammalian organs have been subjected to silicic acid chromatography using light petroleum (b.p. 60–80°) containing increasing concentrations of $CHCl_3$. The fractions eluted with 50% (v/v) $CHCl_3$ in light petroleum contained all the unesterified cholesterol, but on paper chromatography in a reversed-phase system [using paper impregnated with liquid paraffin and $CHCl_3$–MeOH–paraffin (4:5:1, by vol.)], the fractions were shown to contain small amounts of a second compound which stained blue with ethanolic phosphotungstic acid (Martin, 1957). Cholesterol stains pink with this reagent. In some extracts traces of other compounds of low mobility were visible in ultraviolet light.

The second component has been separated from cholesterol on a reversed-phase column (Danielsson, 1958). The material gave a turquoise-blue colour with Lifschütz reagent, prepared according to Bergström & Wintersteiner (1941). This reaction is characteristic of 7-hydroxycholesterol, but since the chromatographic behaviour of the material was consistent with that of a monohydroxy compound, it seemed likely that it was an ester of 7-hydroxycholesterol. Infrared analysis confirmed the presence of an ester group at the C-3, and on mild alkaline hydrolysis material was obtained which was chromatographically identical with 7α-hydroxycholesterol.

A series of 3β-acyl esters of 7α-hydroxycholesterol (lauryl to stearyl) was synthesized using a modification of the method described by Henbest & Jones (1948) for the acetyl ester. These esters were labile and dehydration occurred on alumina columns giving cholestadienes and trienes, while in the presence of ether or on exposure to ultraviolet light, Lifschütz-negative products were formed.

The synthetic esters were chromatographically identical with the isolated material in the three systems described.

The naturally occurring material, which was found in extracts of human and rat serum and in rat skin and liver, may have arisen from cholesterol esters by an oxidation or peroxidation reaction at C-7 (Bergström, 1942).

Experiments with pancreatin powder have shown that identical material can also be produced enzymically by esterification of 7α-hydroxycholesterol with the appropriate fatty acids.

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